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GLUTAMIC ACID DECARBOXYLASE: ISOLATION FROM BOVINE CEREBELLUM AND IMMUNOLOGICAL QUANTITATION IN DEVELOPING MOUSE BRAIN

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IN DEVELOPING MOUSE BRAIN

by

RICHARD ALBERT HADJIAN
B.A. Boston University, 1971

A THESIS

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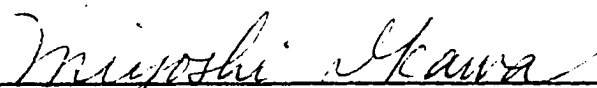
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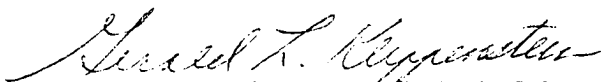
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TO BARBARA

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ABSTRACT

GLUTAMIC ACID DECARBOXYLASE: ISOLATION FROM BOVINE CEREBELLUM AND IMMUNOLOGICAL QUANTITATION IN DEVELOPING MOUSE BRAIN

by

RICHARD ALBERT HADJIAN

Glutamate decarboxylase catalyzes the formation of carbon dioxide and γ -aminobutyric acid from its substrate, L-glutamic acid. γ -Aminobutyric acid is an important inhibitory neurotransmitter in vertebrate and invertebrate nervous systems. L-Glutamate decarboxylase (GAD) is probably the rate limiting enzyme in determining steady state levels of γ -aminobutyric acid in normal nervous tissue. However, due to previously unsuccessful attempts to purify the enzyme to homogeneity, little is known with certainty about the detailed properties of the enzyme.

A chromatographic procedure was developed to purify GAD from bovine cerebellum. However, only a 32-fold increase in enzyme specific activity could be obtained due to the enzyme's lability. Studies were made of the enzyme's thermal stability, pH optimum, inhibition by anions and carbonyl trapping agents, and effect on activity by its coenzyme pyridoxal-5'-phosphate.

The purified enzyme preparation was analyzed on analytical acrylamide disc gel electrophoresis. The GAD band was identified by slicing, eluting, and assaying the gel slices for enzyme activity. The GAD band was then re-electrophoresed at various pH's and percentages of acrylamide to determine homogeneity of the band. A large scale gel procedure was undertaken to collect a sufficient quantity of electrophoretically homogeneous GAD for production of antibodies by New Zealand White rabbits.

Monospecific antisera against bovine GAD were obtained from the rabbits. The antisera against GAD isolated from bovine cerebellum were found to react with the decarboxylase of a bovine cerebellum and mouse brain with a single sharp precipitin line.

GAD enzyme specific activity was examined at various postnatal ages of developing mouse brain. An initial rise in GAD activity occurs at 6 days postnatally followed by a rapid increase in enzymatic activity which reaches a maximum at 28 days postnatally.

A quantitative measurement of the amount of GAD in developing mouse brain was performed. The quantitative immunoprecipitation of GAD by rabbit anti-bovine GAD antisera indicates that the amount of protein in an immune precipitate per brain increases 10-fold over the period between 1-28 days postnatally. Such an increase closely coincides with the GAD enzyme activity profile. Therefore, the increase in GAD enzyme specific activity during the postnatal development of

mouse brain represents an increase in the absolute amount of GAD enzyme protein.

INTRODUCTION

Information processing in nervous systems is in all likelihood a result of a coordinated dynamic interplay between excitation and inhibition among neuronal systems. The presynaptic liberation of compounds that produce either an excitatory or inhibitory effect on the specialized postsynaptic regions of neural membranes is a form of cell:cell communication. The disciplines of electron microscopy, physiology, biochemistry, and pharmacology have documented the existence of chemicals as the basis for cellular communication in the nervous system. Presynaptic vesicles and synaptic clefts at neuronal junctions have been revealed by electron microscopy; a synaptic time-delay, characteristic of chemical diffusion, has been detected by physiological studies; and the demonstration of over a dozen chemicals, endogenous to nervous tissue, that can excite or inhibit neurons has been shown by biochemical and pharmacological studies.

A central concept is that contact at the outer neuronal membrane surface with various chemical substances produces physical changes in the membrane structure resulting in a change in the membrane's permeability to small cations. Experimentally derived theories suggest the excitable membranes affinity for the divalent cation, calcium, decreases via contact with an excitatory transmitter (see review by Koketsu et. al., 1969). Thus, conformational changes in the

membrane ultrastructure caused by a loss of Ca^{2+} permits the entrance of Na^+ and exit of K^+ from the neuron. Depolarization is, therefore, a result of an excitatory effect. The inhibitory substances affect membranes by producing an increase in K^+ or Cl^- conductance, or by an electrogenic Na^+ ion pump. A return to resting potential from the depolarized state is accelerated by this last event.

Data now implicates several naturally-occurring amino acids as putative excitatory or inhibitory transmitters (see review by Kravitz, 1967). Glutamic and aspartic acids may be excitatory transmitters and γ -aminobutyric acid (GABA) and glycine may be inhibitory transmitters. Thus, a comprehensive study of the properties and localization of the enzymes that are involved in the metabolism of these is necessary for forming hypotheses on the mode of action of the transmitters at a molecular level.

The only putative neurotransmitters whose localization in specific brain tracts have been established are the catecholamines, norepinephrine and dopamine, and the indoleamine, serotonin. The histochemical mapping of monoamine neurons in the brain (Hillarp *et. al.*, 1966) has led to the identification of catecholamine and serotonin tracts. Monoamine neuronal tracts occur as several separate and distinct tracts. Norepinephrine neuronal cell bodies occur primarily in the medulla oblongata, pons, and midbrain where their axons either descend in sympathetic columns of the spinal cord or ascend into the medial forebrain, primarily in the

hypothalamus. Serotonin cell bodies are located in the raphe nuclei, a series of nuclei in the lower midbrain and upper pons. Serotonergic axons follow the same pattern as those of the noradrenergic neurons. Dopaminergic neuronal tracts, unlike the preceding examples, are found more widely distributed, although they are highly concentrated in the substantia nigra, caudate nucleus, and putamen. A second dopamine tract occurs in the midbrain and a third in the hypothalamus leading to the pituitary gland.

The rate of biosynthesis of the catecholamines is controlled by a rate-limiting hydroxylation of their precursor, tyrosine, to form dihydroxyphenylalanine (DOPA). Tyrosine hydroxylase is found mainly in the catecholamine nerve terminal and located in the cytoplasm (Nagatsu *et. al.*, 1964). Thus, the initial step in the neurotransmitters' synthesis is outside the synaptic storage vesicles. That tyrosine hydroxylase activity can be inhibited by the catecholamines suggests the regulation of biosynthesis by a feedback inhibition mechanism (Udenfriend, 1966). This implies that catecholamine synthesis would be enhanced to replace those which were discharged by nerve stimulation. Results of Alousi *et. al.* (1966) indicate that the stimulation of peripheral sympathetic nerves rapidly increases the formation of norepinephrine from tyrosine. Norepinephrine synthesis was not enhanced by addition of exogenous DOPA, thus indicating that the initial step is the stimulated one. These results are consistent with the interpretation that tyrosine

hydroxylase activity is stimulated by a release of feedback control without an increase in enzyme quantities. However, extended stress activates the synthesis of more enzyme (Alousi *et. al.*, 1966).

Aromatic-L-amino acid decarboxylase (DOPA decarboxylase) converts DOPA to dopamine. It too is located in the cytoplasmic region of the catecholamine nerve terminal (Rodriguez *et. al.*, 1964). Dopamine is subsequently hydroxylated by dopamine- β -hydroxylase, a copper requiring enzyme (Kaufman *et. al.*, 1965), whose locale is unestablished, to norepinephrine. This enzyme is lacking in dopaminergic neuronal tracts where norepinephrine is present in negligible concentration (Stjarne and Lishajko, 1967).

Catabolism of catecholamines is governed primarily by two enzymes. Monoamine oxidase (MAO) oxidatively deaminates dopamine or norepinephrine to aldehydic compounds; catechol-O-methyl transferase (COMT) methylates the catecholamine hydroxyl group in the meta position by the transfer of a methyl moiety from S-adenosylmethionine (Axelrod, 1965). Methylation by COMT may also proceed on the aldehyde compounds formed by MAO. Thus, both MAO and COMT are non-specific monoamine degrading enzymes.

Dopamine is, specifically, methylated by COMT to 3-O-methyl dopamine, which is deaminated to 4-hydroxy-3-methoxy phenylacetic acid (homovanillic acid:HVA). The measurement of HVA in the cerebrospinal fluid is used as an indicator of the activity of dopaminergic neurons. Degradation of nor-

epinephrine proceeds either by COMT to normetanephrine or by MAO to a glycoaldehyde. The glycoaldehyde is then reduced by alcohol dehydrogenase to a glycol, or oxidized by aldehyde dehydrogenase to mandelic acid. These by-products are then methylated by COMT; the glycol compound is converted to 3-methoxy-4-hydroxyphenylglycol (MHPG) in the central nervous system (CNS); mandelic acid to an O-methylated acid, vanillylmandelic acid (VMA) in the peripheral nervous system. VMA in urine is measured as a clinical index of sympathetic nervous function.

The functioning of the two major transmitter degrading enzymes, COMT and MAO, is elucidated by their subcellular localizations. MAO is found in the outer mitochondrial membranes of the catecholaminergic nerve terminal. Thus, catecholamines that either leak out of storage vesicles into the cytoplasm or are taken up from the synaptic cleft are metabolized before acting on postsynaptic sites (Axelrod, 1965). Although COMT distribution appears to correlate with endogenous catecholamines (Axelrod *et. al.*, 1959), the enzyme does not appear to be present in catecholamine-containing nerve terminals, since COMT activity is not reduced after sympathectomy (Potter *et. al.*, 1965). This is consistent with the interpretation that extraneuronal COMT acts on norepinephrine discharged into the synapse, but not that norepinephrine inactivated by the reuptake process (see review by Snyder, 1967).

Synaptically discharged catecholamines are inactivated

by reuptake into presynaptic nerve terminals, not by the method of enzymatic degradation (Iversen, 1967). The catecholamine reuptake process was discovered by Axelrod (1965) when intravenously injected radioactive norepinephrine was found in synaptic vesicles. Metabolic inhibitors and low temperatures depress reuptake. The process functions optimally at physiological concentrations of Na^+ and K^+ , is saturable, obeys Michaelis-Menten kinetics, and is inhibited by ouabain, an inhibitor of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Tissari et. al., 1969). The reuptake-storage process may be disrupted by the drug reserpine (Euler and Lishajko, 1963), which releases norepinephrine from the presynaptic catecholaminergic vesicles (Iversen, 1967), or by the tricyclic antidepressant imipramine which blocks reuptake from the synaptic cleft (Snyder, 1967).

Serotonin (5-hydroxytryptamine) is formed from an essential amino acid, tryptophan. Tryptophan is first hydroxylated to 5-hydroxytryptophan (5-OH-trp) by tryptophan hydroxylase (Ichiyama et. al., 1970), which unlike tyrosine hydroxylase, has not been clearly established as a rate-limiting enzyme. 5-OH-trp is then decarboxylated to serotonin by aromatic-L-amino acid decarboxylase which may be the same enzyme as DOPA decarboxylase (Lovenberg et. al., 1962; Udenfriend et. al., 1970 and 1971). The evidence for feedback inhibition on tryptophan hydroxylase by serotonin is weak (Eiduson, 1966).

The inactivation of serotonin is similar to the pro-

cess for catecholamines, that is by a specific reuptake system operating at the level of the neuronal membrane. This reuptake process was first thought to be unique for the catecholamines, however it now appears to be a universal method for inactivating compounds after they have acted as neurotransmitters, and that acetylcholine's enzymatic breakdown is the unusual situation. Acetylcholine is removed, after its interaction with the postsynaptic receptor site, by a rapid hydrolysis via acetylcholinesterase.

Evidence for glutamate, glutamine, and aspartate as neurotransmitters is still incomplete. Early studies demonstrated that D-glutamate and D-aspartate were both potent inducers of spreading cortical depression in the rabbit, and contraction of crustacean muscle (Van Harreveld, 1959). At present, however, the rationale for a transmitter role at primary sensory nerve endings in the spinal cord rests on (1) a greater concentration of glutamate in dorsal rather than ventral regions of the spinal cord (Duggan and Johnston, 1970), and (2) an excitatory effect by direct application of glutamate to spinal motor neurons (Krnjevic, 1965). The fulfillment of specific criteria for a neurotransmitter: synthesis, storage, release, postsynaptic action, and inactivation are either general or unestablished.

It is possible that the amino acids, glutamate, glutamine, and aspartate may serve as a link between metabolism and function in nerve cells (Huggin *et. al.*, 1967). During the early and rapid growth phase of the brain in both cat

(Berl et. al., 1963) and rat (Bayer et. al., 1967), glutamate concentration increases two-to threefold, while glutamine remains fairly constant. The synthesis of glutamate and glutamine is a major pathway for reducing local levels of ammonia (Krebs, 1935), (Berl et. al., 1962). The major cause of the encephalopathy associated hepatic disease is an elevation of blood ammonia levels. Hyperammonia in blood has been shown experimentally to be epileptogenic and to produce coma rapidly (Schenker et. al., 1965). Review articles showing the possible correlation of the metabolism and function of these compounds have been written (Waelsch, 1951; Curtis et. al., 1965; and Curtis et. al., 1970).

Although glycine is simple structurally, its intermediary metabolism and functional role in the nervous system is complex. Glycine has been shown to be present in large amounts in the CNS (Aprison et. al., 1968), and pharmacological and electrophysiological evidence support its role as an inhibitory transmitter in the spinal cord of cat (Werman et. al., 1968). Evidence is accumulating for its presynaptic release from isolated perfused (^{14}C -glycine) toad spinal cord during stimulation of the dorsal roots (Aprison, 1970). Also, a relationship exists between the number of neurons involved in polysynaptic inhibitory reflexes and glycine content (Davidoff et. al., 1967). Work with the convulsant, strychnine, further supports glycine's inhibitory role, since by its normal blocking of the postsynaptic inhibition of motor neurons, strychnine antagonizes

the action of glycine. Presumably strychnine interferes with postsynaptic membrane's receptor site for glycine (Curtis et. al., 1968). A high-affinity transport process for glycine from the synaptic cleft has been found in spinal cord (Neal et. al., 1969).

GABA is an established inhibitory transmitter at the crustacean neuromuscular junction (Kuffler et. al., 1958) where it is almost exclusively localized within inhibitory axons and nerve cell bodies (Otsuka et. al., 1967). GABA levels are 100 times greater, and glutamic acid decarboxylase (GAD) (EC 4.1.1.15) is 10 times higher in inhibitory axons and nerve cell bodies, than in corresponding excitatory neurons (Kravitz et. al., 1965). Evidence suggests that GABA may affect the release of transmitter from excitatory nerve terminals and cause an increase in Cl^- permeability at the postsynaptic membrane (Takeuchi et. al., 1966).

Although evidence is less complete, GABA is probably a major inhibitory transmitter in the vertebrate nervous system (Roberts et. al., 1968), as well as the invertebrate. Applied directly to the brain of mammals GABA has a non-specific depressant action (Bhargava et. al., 1964). Its regional distribution in vertebrate brain is compatible with its inhibitory transmitter role; in rabbit cerebellum GABA concentration is highest in the inhibitory Purkinje cells (Kuriyama et. al., 1966).

The formation and metabolism of GABA in the nervous system involves a major pathway in the brain. In the CNS

of vertebrate organisms GABA is formed by an irreversible decarboxylation of L-glutamic acid. GAD catalyzes this reaction requiring pyridoxal-5'-phosphate (PyP) as a co-enzyme. Regional levels of GAD accurately reflect regional levels of GABA not only in guinea pig brain, but also in other species (Sisken et. al., 1960). Such a reliable correlation between enzyme activity and substrate concentration substantiates the concept of GAD as being the major pathway to GABA in nervous tissue.

GABA degradation is via a reversible transamination with α -ketoglutarate (α -KG) as the amino group acceptor. The reaction is catalyzed by GABA- α -ketoglutarate transaminase (GABA-T) to form glutamate and succinic semialdehyde (SSA). GABA-T is a pyridoxal phosphate requiring enzyme, as is GAD (Baxter et. al., 1958) and is found chiefly in the gray matter of the CNS (Fahn et. al., 1968), (Salvador et. al., 1959). In contrast to GAD, GABA-T appears to be a mitochondrially-bound enzyme, of which 20% of its activity resides in the presynaptic nerve terminal (Salganicoff et. al., 1965), (Reijnierse et. al., 1975). Intraperitoneally injected U-(¹⁴C)GABA is rapidly metabolized and label is found in respired CO₂, tricarboxylic acid (TCA) cycle intermediates, and related amino acids (Wilson et. al., 1959). The final reaction of GABA degradation is the conversion of SSA to succinate, which enters into the TCA cycle, via succinic semialdehyde dehydrogenase. The enzyme has been studied in detail in humans (Embree et. al., 1964) and has a local-

ization similar to that of GABA-T, that is in the mitochondria of all neuronal regions (Miller et. al., 1967). These individual enzymatic steps constitute the "GABA shunt", which represents an alternative pathway to the portion of the tri-carboxylic acid cycle between α -KG and succinate. The net effect of the shunt is the conversion of α -KG to succinate with the evolution of CO_2 and the reduction of NAD. However only three ATP equivalents are generated by the shunt as compared to four ATP equivalents by the Krebs cycle. In guinea pig cortical slices the "GABA shunt" is completely dependent on the presence of GAD and GABA.

Presynaptic release of GABA has been demonstrated by stimulation of inhibitory axons to various lobster muscles (Otsuka et. al., 1966). Stimulation of excitatory axons have no effect on GABA release. GABA release in vertebrate nervous system has been more difficult to establish. However, its release has been found from the posterior lateral gyri of cats during synaptic inhibition (Mitchell et. al., 1969). Release of ^3H -GABA from pre-loaded cortical slices of rat brain has also been effected by high K^+ concentration (Srinivasan et. al., 1969).

An ionic basis for the inhibitory mechanism of action of GABA on presynaptic membranes of invertebrate and vertebrate neurons has been postulated (Roberts et. al., 1968 and 1960). GABA acts at the presynaptic membrane of crayfish by increasing the permeability to Cl^- (Dudel et. al., 1961) thereby inhibiting the release of excitatory neurotransmitters

(Takeuchi et. al., 1966).

Inactivation of GABA at the synaptic cleft probably proceeds by a transport to intracellular sites. Enzymatic degradation of GABA is unlikely since GABA-T has a intracellular mitochondrial location and thus is not close to the synaptic membrane. One theory postulates a high-affinity uptake of free extraneuronal GABA at membranes and requires a high Na^+ (0.1 M) environment (Roberts et. al., 1968). It is proposed that GABA is transported to the intracellular medium by a carrier-mediated transport system utilizing an active Na^+ gradient coupled to a utilization of ATP. This uptake system would serve to terminate the action of GABA by the removal of GABA from sites of action in the synapse and its subsequent mitochondrial metabolism (Roberts et. al., 1968).

The purification and isolation of mammalian GAD from brain extracts has remained a difficult task because of its extremely unstable nature. A major purification has been accomplished by using buffers containing PyP at 0.1 mM and aminoethylisothiuronium bromide at 1 mM (Susz et. al., 1966). This results in a protection of sulfhydryl and/or other labile reactive groups on the apoenzyme. The only proven procedure uses 9,000 mouse brains to obtain a crude mitochondrial fraction followed by fractionation on DEAE-Sephadex, Sephadex G-200, $(\text{NH}_4)_2\text{SO}_4$ fractionation and calcium phosphate gel chromatography. This procedure has resulted in a 600-700 fold purification of GAD (Wu et. al., 1973; Roberts et. al., 1963). The enzyme's pH optimum varies during the pur-

ification from 6.4 at the first stages, to 7.2 at the final stage; likewise the K_m changes from 0.003 to 0.008 (Wu et. al., 1973). Both of these shifts suggest conformational changes in the protein during the purification procedure. The enzyme remains moderately stable at temperatures below 40° C.

Mammalian GAD can be inhibited both in general and specific manners. Halogen anions such as Cl^- inhibit the protein at 50 mM concentrations, suggesting that there could be regulation of the enzymatic activity by the ratio of glutamate to GABA at particular inhibitory neural junctions (Roberts et. al., 1968). Sulfhydryl reagents and oxidation are potent inactivators of mammalian GAD in vitro (Roberts et. al., 1963), however this may be prevented by SH-protective agents such as glutathione. Estrogens, salicylate, and phenolic compounds are also inhibitors of GAD (Tashian, 1961).

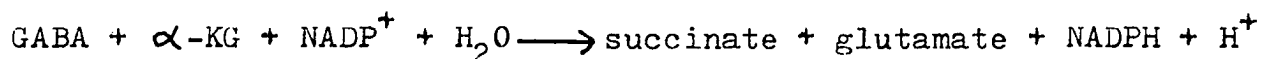
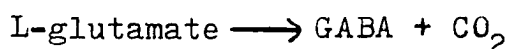
On the basis of available data for GAD and other pyridoxal phosphate-dependent enzymes hypotheses have been made on the coenzyme's linkage to GAD. This information suggest an aldimine linkage of pyridoxal phosphate to two ϵ -amino groups of lysine (Roberts et. al., 1964). Compatible with this theory are both spectral data and in vitro work with PyP analogues and carbonyl trapping agents. The carbonyl trapping agents presumably interact with the aldehyde group of PyP and possibly with amino acid residues of the apoenzyme. Specific carbonyl trapping agents such as hydroxylamine and aminooxyacetic acid inhibit GAD only in vitro; in vivo they

potentiate GAD inhibition by pyridoxal hydrazone formation (Tapia et. al., 1967). Hydrazine, hydroxylamine, and pyridoxal semicarbazone are 100 times more potent inhibitors of pyridoxal kinase, which mediates the synthesis of PyP from pyridoxal and ATP (McCormick et. al., 1960), than GAD in vitro. Thus, if free PyP were reduced (64% of control) by inhibition of pyridoxal kinase, the activity of GAD with its relatively weak affinity for PyP would be affected. Tapia (1973) has shown a correlation between a decrease in PyP and GAD activity (25% of control) after administration of pyridoxal-5'-phosphate- γ -glutamyl hydrazone (PyPGH). Tapia (1969) suggests that after PyPGH treatment the following sequence of events occurs in the nerve ending: inhibition of pyridoxal kinase---decrease of levels of PyP---inhibition of GAD---decreased rate of GABA synthesis---convulsions. Thus a decrease in the critical concentration of PyP would result in less GABA, the putative inhibitory transmitter.

GAD activity has been determined by either the rate of CO₂ release or the rate of GABA formation. The evolution of CO₂ is usually measured by manometric, spectrophotometric, or isotopic techniques. GABA formation rates have been measured by two-dimensional paper chromatography and high-voltage paper electrophoresis (Smith, 1960) or enzymatically (Hakkinen and Kulonen, 1963). However, none of these methods facilitate the handling of large numbers of samples.

Cozzani (1970) developed a spectrophotometric assay for a kinetic study of GAD from Clostridium perfringens. The

assay is accomplished by the addition of the "Gabase System", GABA transaminase coupled to succinic semialdehyde dehydrogenase. The actual sequence of the coupled enzyme reaction is:



Thus, the velocity of the enzymatic removal of the α -carboxyl group from L-glutamate is estimated from the rate of reduction of NADP^+ .

An automated P_{CO_2} (partial pressure) assay for GAD (Zeman et. al., 1973) has been developed which is based on the measurement of the partial pressure of CO_2 in a reaction mixture as a measure of total CO_2 formed by enzymic decarboxylation of glutamate.

CO_2 liberated at acidic pH is usually measured by conventional manometric techniques in a Warburg apparatus. First described in 1951 by Roberts and Frankel the method uses a high specific activity radioactive substrate and the measurement of liberated $^{14}\text{CO}_2$. The technique has undergone refinements and is now the method of choice for the measurement of GAD enzyme activity. Under the proper conditions (Roberts and Simonsen, 1963), the technique allows for essentially complete absorption of $^{14}\text{CO}_2$ by Hyamine.

The original method for absorbing and counting $^{14}\text{CO}_2$ by liquid scintillation spectrometry (Passman *et. al.*, 1956) was modified for use with substrates of high specific activity to detect as little as 10^{-11} moles of CO_2 . This microradiometric technique was established by Albers and Brady (1959) to permit analysis of large numbers of samples with a minimum of manipulation. The vessels for this reaction have been modified repeatedly (Wilson *et. al.*, 1972; Moskal and Basu, 1975). The assay method for the experiments presented in this thesis are discussed in the Methods section.

Comparative studies with GAD from crustacean nervous tissue show both similarities to and differences from the mammalian enzyme (Molinoff *et. al.*, 1968). While the mouse enzyme is not affected by a feedback regulation by GABA, the lobster enzyme is inhibited by GABA (K_i calculated as 1.25×10^{-3} M GABA, Roberts *et. al.*, 1963). This feedback regulation may control the production of GABA at lobster inhibitory synapses while Cl^- may fill this role in the mammalian system (Roberts *et. al.*, 1968). Halogen anions do not affect lobster GAD, but K^+ is required, unlike the mammalian enzyme.

The developmental relationship between GAD and GABA have been examined in the brains of mice, rats, chickens, cats, dogs, and bullfrogs. In all these systems, and within specific brain areas, enzyme and substrate levels progressively increase until mature levels are reached. The age of

increases in both GAD activity and GABA levels coincide with the period of rapid growth of the surface area of cortical dendrites in rabbit (Agrawal et. al., 1967).

Regional distribution of GAD in the CNS of several mammalian species, including man, has been studied (Muller et. al., 1962). Autoradiographic distribution patterns of 2-¹⁴C-GABA, injected stereotaxically into rats, indicate that areas of gray matter contain the highest levels of GAD. In rabbit, GAD is found in the inhibitory synapses of the Purkinje cell layer (Kuriyama et. al., 1966). The dorsal gray matter of cat has twice the GAD level as the ventral region (Graham et. al., 1969).

Data regarding the subcellular distribution of GAD can be perplexing. Differential centrifugation of crude brain homogenates indicates GAD migrates with the crude mitochondrial fraction (Shatunova et. al., 1964). However, osmotic shock which releases the GAD from the particulate fraction without irreversibly damaging mitochondria indicates that GAD is a cytoplasmic enzyme localized mainly in the presynaptic nerve terminal axoplasm (Salganicoff et. al., 1965). It also appears that the amount of GAD released by osmotic shock is Ca^{2+} dependent. A concentration of 4 mM CaCl_2 prevents substantial release of GAD into the supernatant fraction. The quantity of GAD released increases when a chelating agent for Ca^{2+} , or a membrane dispersant such as Triton X-100 are used (Salganicoff et. al., 1965). These results on the fixation of the enzyme by Ca^{2+} suggest that

the Ca^{2+} present in nerve endings is probably sufficient to promote GAD binding to the outer surface of synaptic vesicles. Thus, GABA formed may diffuse into the axoplasm or be collected within the synaptic vesicle. This situation would be similar to the synthesis of acetylcholine by choline acetylase, an enzyme attached to the synaptic vesicle's surface (Ritchie and Goldberg, 1970). Immunocytochemical localization of GAD in developing rodent cerebellum indicates the enzyme is in close association with synaptic vesicles and at presynaptic junctional membranes (McLaughlin *et. al.*, 1975).

The present work describes an isolation procedure for bovine cerebellum GAD via column chromatography and acrylamide gel disc electrophoresis. Monospecific antibodies for GAD were obtained, and subsequently quantitative measurements of the amount of GAD in developing mouse brain were performed. This allowed us to determine if the increase in GAD activity during development was due to an increase in the quantity of enzyme, or an enhancement in activity of existing enzyme.

MATERIALS AND METHODS

Materials

The mice in all developmental experiments were C57BL/6J, originally obtained from Jackson Laboratories, Bar Harbor, Maine, and maintained by brother x sister matings. Food (Charles River) and water were available ad libitum; mice were maintained with a 14 hour light, and 10 hour dark period. The temperature of the mouse facility is $72^{\circ} \text{ F} \pm 2^{\circ}$ with a relative humidity of $50 \pm 5 \%$.

Bovine brains were obtained from the Granite State Packing Co., Manchester, New Hampshire; human brains were obtained from the Massachusetts General Hospital, Boston, Massachusetts. Bovine brains were removed as soon as possible after death of the animal and stored on ice until returned to the laboratory. Meningeal membranes were removed from the brains and brains were stored at -20° C .

New Zealand White rabbits were obtained from Camm Research Institute, Wayne, New Jersey. Food (Agway, Inc.) and water were available ad libitum; rabbits were maintained similarly to mice.

All chemicals used were reagent grade.

Glutamate Decarboxylase Assay

A modification of the method of Wilson et. al., 1972, was employed. Substrate, L-(1- ^{14}C)glutamic acid, was ob-

tained from Calatomic, Inc., Los Angeles, California and stored at -20°C prior to use. Solid L-(1- ^{14}C)glutamic acid was dissolved in 2.5 ml of 50 mM potassium phosphate buffer, pH 6.8, 1 mM dithiothreitol (DTT) prior to use. Non-radioactive glutamic acid was added to give a specific activity of 20 mCi/mmole. Each reaction contained the following components in a final volume of 100 μl : 10 μl , 5 mM L-(1- ^{14}C)-glutamic acid (1 μCi per reaction) dissolved in 50 mM potassium phosphate, pH 6.8, 1 mM DTT (buffer A); 10 μl , 1 mM pyridoxal-5'-phosphate (Sigma) in buffer A; 80 μl , brain homogenate or a dilution thereof. Reaction mixtures were sealed in 17 x 120 mm conical glass centrifuge tubes with tightly fitting rubber stoppers holding a plastic cup (Ace Glass). The plastic cup held a number 0 gelatin capsule (Lilly) containing 0.2 ml of 1 M Hyamine hydroxide in methanol. Addition of the reaction components was done with the vials kept on ice. Reaction tubes were then transferred to a shaking water bath and incubated for 30 min at 37°C . Reactions were stopped by injection of 100 μl of 2 M glacial acetic acid through the rubber stopper; complete absorption of $^{14}\text{CO}_2$ by the Hyamine solution was allowed for 30 min. The rubber caps were then removed and the gelatin capsules removed with forceps and placed in a scintillation vial containing 10 ml of toluene-fluor scintillation mixture.

The toluene and fluors solution was prepared by dissolving 50 grams PPO (2,5-diphenyloxazole) and 0.625 grams POPOP (p-bis(5-phenyloxzoly1-2-)-benzene) in 500 ml of reagent

grade toluene. The final liquid scintillation solution was prepared by adding 42 ml of the above solution to 1 liter of toluene. Background fluorescence was reduced by addition of 1 ml glacial acetic acid to this solution. Radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation counter. Where noted counts have been left as CPM, since the efficiency of ^{14}C counting as determined by the external standard method remained constant.

Glutamate Decarboxylase Partial Purification

Preparation of Starting Material

Bovine Cerebellum was thawed and homogenized (Osterizer blender) in buffer A (2 ml per gram wet weight). The homogenate was centrifuged at $35,000 \times g$ for 20 min at 4°C in a Sorvall centrifuge. The supernatant was dialyzed overnight at 4°C . At each stage in the protocol a protein determination (Warburg and Christian, 1942) and a GAD assay was performed.

First DEAE-Sephadex Column

The dialyzed sample (see Fig. 2 for details) was loaded onto a DEAE-Sephadex A-50 column (2.5 x 50 cm) that had been previously equilibrated with buffer A. An equal volume of buffer A was introduced after the sample. A linear gradient elution system consisting of 250 ml of 0.1 M potassium phosphate, pH 6.8, 1 mM DTT, and 250 ml of 0.6 M potassium phosphate, pH 6.8, 1 mM DTT was used. The flow

rate of the effluent, 0.5 ml per minute, was kept constant by the use of a peristaltic pump and 4 ml fractions were collected. All work was done at 4⁰ C. The highest specific activity fractions were pooled and dialyzed overnight against buffer A.

Second DEAE-Sephadex Column

DEAE-Sephadex A-50 (2.5 x 50 cm) was equilibrated with buffer A. The enzyme solution from the preceding step was then applied (see Fig. 3), followed by an equal volume of buffer A. The batchwise elution system consisted of the following buffers:

1. 100 ml, 0.1 M potassium phosphate, pH 6.8; containing 1 mM DTT,
2. 400 ml, 0.2 M potassium phosphate, pH 6.8; containing 1 mM DTT.

High activity fractions were pooled and dialyzed against 2 volumes of buffer A overnight with one change.

PM-30 Concentration

The dialyzed sample was concentrated to approximately 14 ml under N₂ (30 psi) using a PM-30 Diaflow pressure ultra-filtration membrane (Amicon).

Gel Filtration on a Sephadex G-100 Column

The concentrated enzyme was applied to a Sephadex G-100 column (1.5 x 100 cm) previously equilibrated with buffer A. The column was eluted with buffer A at a flow rate of 10 ml

per hour, kept constant by the use of a peristaltic pump. 4 ml fractions were collected. Highest specific activity fractions were pooled and aliquots stored at -20° C.

Isolation and Radioactive Assay of GAD on Polyacrylamide Gels

Electrophoresis was performed in a Hoeffer apparatus using a discontinuous buffer system (Davis, 1964). The standard anodic gel system stacking at pH 8.9 and running at pH 9.5 was used, with the only exception being the substitution of a Tris-phosphoric acid buffer in place of the usual Tris-HCl. Separating gels were initially run for one hour in a continuous buffer system (a 1:8 dilution of Tris-phosphoric acid buffer) to eliminate oxidation contaminants of gel polymerization. Gels were run at the following percentages of acrylamide, 3.5, 5.25, 7.0, 10.5, with the 3.5% gel providing the clearest separation of the GAD band from other proteins. Tubes were 12 cm long and 5 mm inside diameter, and contained 5 cm of separating gel and 0.5 cm of stacking gel. The gels were run at constant current of 1.5 mA/gel at 4° C. Bromophenol blue was used as a tracking dye and the gels were run until the dye front was 5 mm from the end of the gel; the gels were removed and the dye front marked with a wire. The gels were then quick-stained with Buffalo Black solution at 90° C for 20 min, destained electrophoretically for 20 min in 7 % acetic acid, and left in a diffusion destainer (in 7 % acetic acid) until all background staining was eliminated. Quantitation of the amount

of GAD protein was done by running known concentrations of bovine serum albumin on gels and comparing densitometric integrator units to those obtained for GAD as determined by a Joyce-Loeble densitometer.

The determination of the migration position of GAD in polyacrylamide gels was accomplished by enzyme assay of slices from unstained gels. After electrophoresis the proteins were visualized by immersing gels in a 0.003 % solution of 1-anilino-8-napthalene sulfonate (ANS) (Hartman et. al., 1969) under a ultraviolet lamp. The distance of bands from the dye front was measured. Another gel was cut, starting at the dye front, into 2-mm slices. Each slice was placed into GAD assay tubes containing 0.5 ml, 0.1 M potassium phosphate, pH 6.6, 1 mM DTT, and 1 mM PyP. They were shaken in an ice bath for 2 hours and frozen at -20° C. After thawing 10 μ Ci of L-(1- 14 C)glutamic acid was added to each gel slice solution, followed by a 60 min incubation at 37° C. Enzyme activity coincided with one major protein band, as positioned by ANS.

Re-electrophoresis of GAD was performed by homogenizing (Duall homogenizer, Kontes Glass Co.) two gel slices in 0.37 ml of buffer A, followed by electrophoresis as described above, at multiple pH's and percentages of acrylamide.

Rabbit Immunization and Immunochemical Analysis

Rabbits were injected with electrophoretically pure GAD. The following injection protocol was followed: 250

μ g of homogenized GAD protein contained in the gel slices was emulsified with 6 ml of Freund's complete adjuvant (Miles Laboratories, Inc.); Injections were distributed among 5 intradermal sites in the back, hind toe pads, and thigh muscles; 250 μ g suspensions in Freund's incomplete adjuvant were injected intramuscularly on the 10th and 20th day; a 100 μ g injection was given on day 30. Rabbits were bled 10 days after the final injection. Rabbits were bled by ear vein periodically to determine the extent of antibody production. Sera were collected by allowing the blood to coagulate at 4° C then pouring off the clear amber sera and storing it at -20° C. Monospecificity of the rabbit anti-bovine-GAD sera was tested on micro double immunodiffusion plates (Hyland, pattern C). Antisera were tested against bovine and mouse brain homogenates, and the most highly purified bovine GAD. Development of plates was made at 4° C.

Quantitative Immunoprecipitation of Mouse GAD

Mice at various postnatal ages were killed by decapitation, their brains removed, weighed, and stored at -20° C. The brains were homogenized in a Duall homogenizer in buffer A (2 ml per gram wet weight), centrifuged at 35,000 x g for 20 min at 4° C. The supernatant was assayed for GAD activity and utilized for quantitative immunoprecipitation.

The following assay procedure was utilized: increasing concentrations of immune sera between 20-100 μ l were diluted to a constant volume of 100 μ l with distilled water, to

which a constant volume (15 μ l) of the mouse brain 35,000 x g supernatant fraction was added. The solutions were shaken for 60 min first at room temperature, then at 4° C. They were centrifuged (International, Model PR-2) at 1,000 RPM for 10 min at 4° C to pellet the antigen-antibody complex. The 1,000 RPM supernatant (80 μ l aliquot) was assayed for GAD activity. The pellet was washed 3 times with buffer A and its protein concentration determined by the Lowry method, modified by Oyama and Eagle, (1956).

For developmental studies mouse brain 35,000 x g supernatant fractions from various ages were adjusted to 4 mg protein/ml. Quantitation of GAD by immunoprecipitation was performed essentially as described above, with the following modification: increasing concentrations of immune sera between 10-70 μ l were diluted to a constant volume of 70 μ l with distilled water, to which 50 μ l of a brain extract was added.

RESULTS

Standard Assay

The GAD enzyme activity assay was based on the evolution, and subsequent absorption of $^{14}\text{CO}_2$ by a Hyamine hydroxide solution. The efficiency of ^{14}C counting by the external standard method was constant at 90-94% efficiency, therefore all counts were left as CPM. The use of gelatin capsules to hold the Hyamine solution does not affect the efficiency of ^{14}C counting. The decarboxylation of radioactive substrate (L-(1- ^{14}C)glutamic acid) was first studied to establish its linearity with respect to protein concentration of a bovine brain extract. The extract used was prepared as described in Methods. Figure 1 indicates that GAD activity is proportional to protein concentration over a thirty-three fold range. This linear increase in GAD activity with respect to protein concentration was highly reproducible and re-checked for all extracts studied to insure the validity of the assay method.

Partial Purification of GAD

In a typical preparation 200-250 ml of a crude bovine cerebellum extract was dialyzed overnight versus an excess of standard buffer A to reduce endogenous levels of brain glutamate. This dialyzate was put on a DEAE-Sephadex A-50 column. Fractions were assayed for A_{280} and GAD activity.

Figure 2 shows the A_{280} and GAD activity of the eluant

Fig. 1. GAD enzyme activity with respect to protein concentration of a bovine brain extract

The bovine cerebellum extract was prepared as described in Methods.

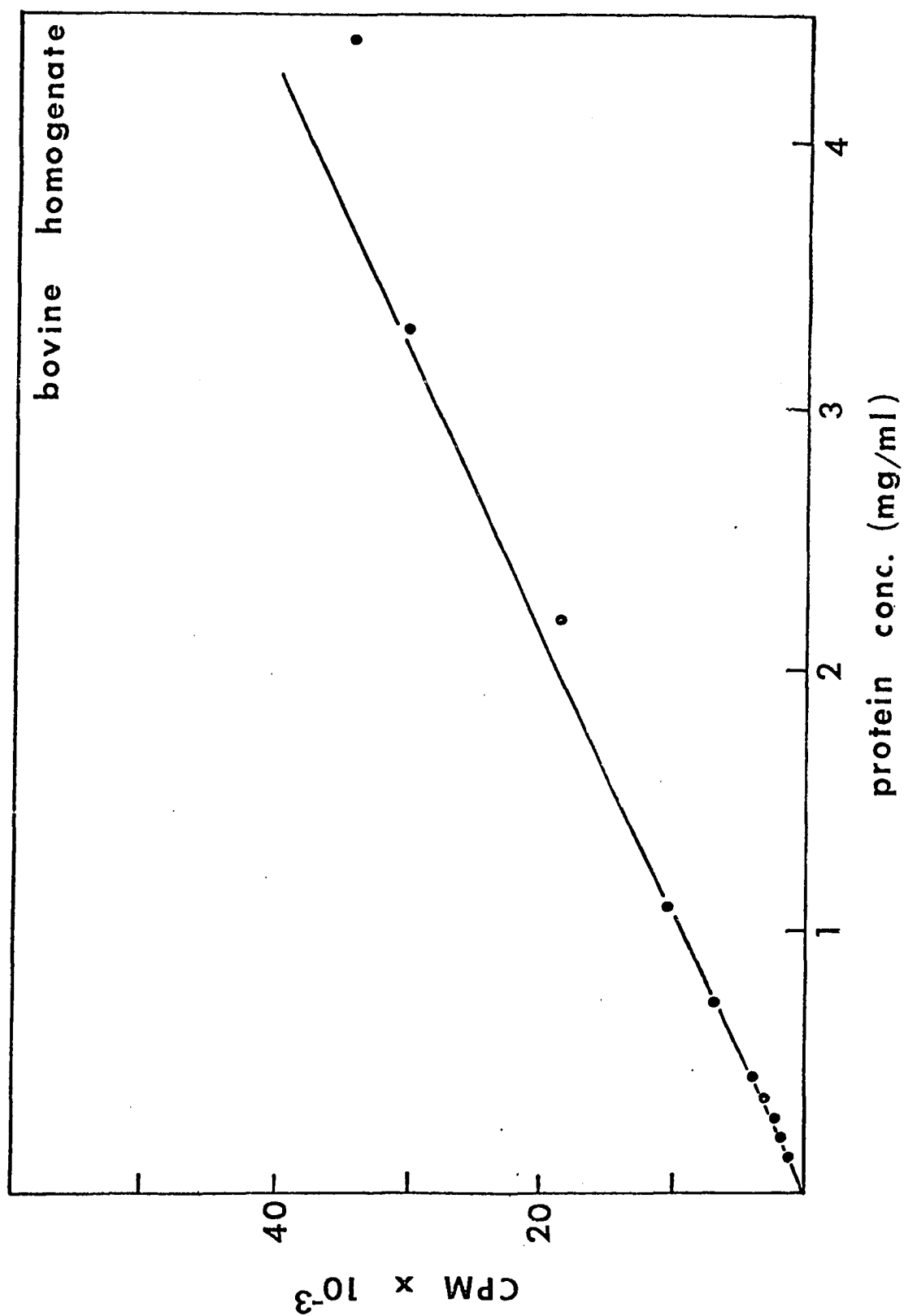
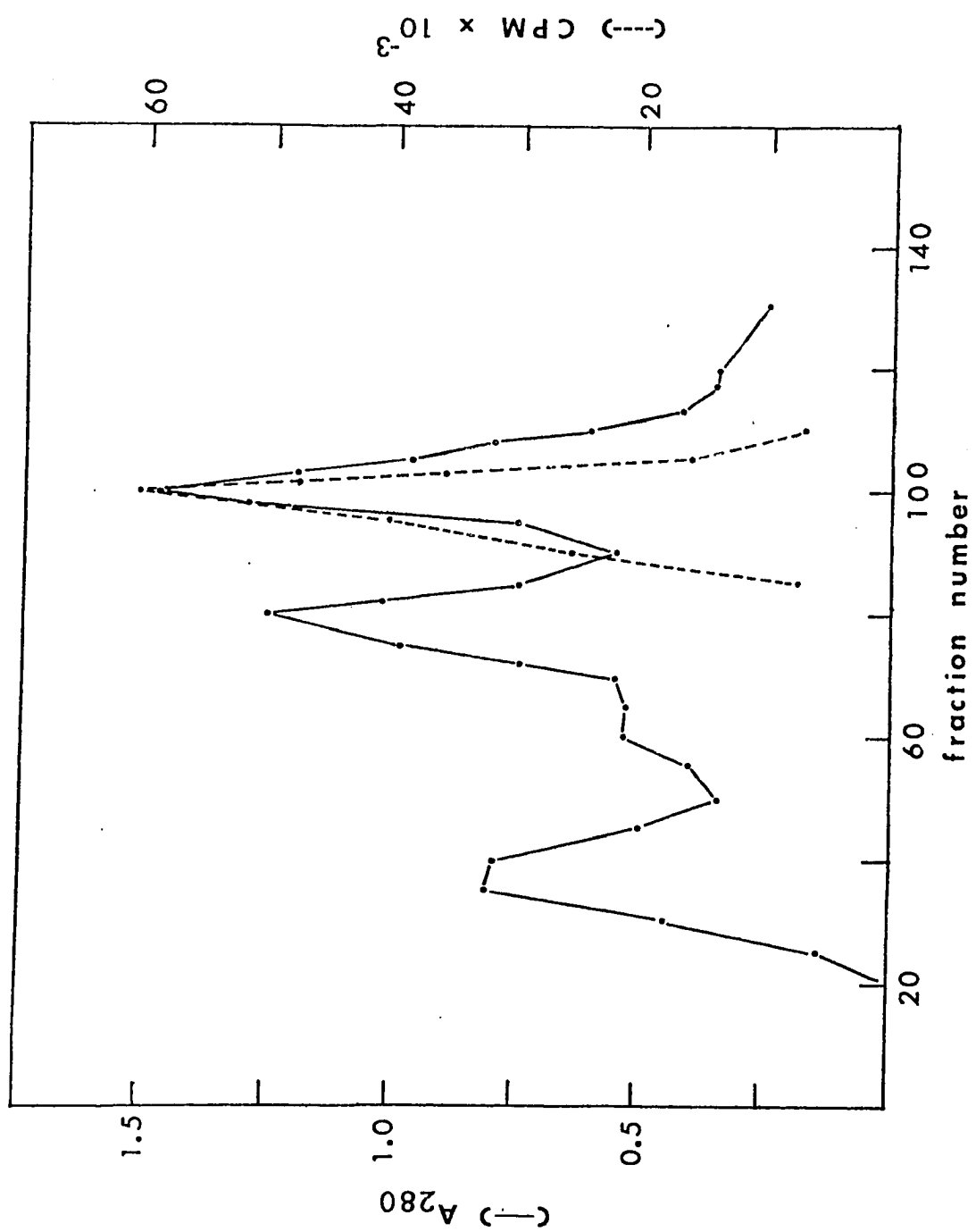


Fig. 2. Elution profile of the first DEAE-Sephadex column

220 ml of dialyzed bovine cerebellum extract was put onto a DEAE-Sephadex A-50 column (2.5 x 50 cm). The column was eluted with a linear gradient between 0.1-0.6 M potassium phosphate (pH 6.8), 1 mM DTT, at a flow rate of 30 ml/hr and 4 ml fractions were collected. A_{280} (—); GAD activity (---).



from the first DEAE-Sephadex column. The bulk of the A_{280} material elutes within the two initial peaks, between fractions 20 and 90, while all the GAD activity elutes in a single peak at approximately 0.2 M phosphate buffer. Fractions showing the highest GAD specific activity were pooled and dialyzed overnight to reduce phosphate concentration to that of the standard buffer (50 mM). This initial procedure yields a 16-fold purification of the enzyme.

The dialyzed fraction, 50-75 ml, was then loaded onto the second DEAE-Sephadex A-50 column. Figure 3 show the A_{280} and GAD activity of the eluant from the second DEAE-Sephadex column. The bulk of the A_{280} material elutes in the first peak and GAD is found exclusively in the second peak around fraction number 125. The fractions with the highest GAD activity were pooled, dialyzed overnight, and concentrated using a PM-30 Diaflow ultrafiltration membrane.

The PM-30 concentrate, 10-15 ml, was put on a Sephadex G-100 column (1.5 x 100 cm). Figure 4 shows the A_{280} and GAD activity profile of the eluant of the Sephadex G-100 column. Protein eluted in two approximately equal peaks and GAD activity was found midway between these two peaks. GAD activity, which eluted around the fourteenth fraction, was pooled and this fraction was labeled as partially purified bovine GAD.

Table 1 shows that the chromatography protocol results in an approximate 32-fold purification of the bovine enzyme and approximately a 7% recovery of starting GAD enzyme activity.

Fig. 3. Elution profile of the second DEAE-Sephadex column

65 ml of the dialyzed pool from the first column was put onto a second DEAE-Sephadex A-50 column (2.5 x 50 cm). The column was eluted with a batchwise elution system, 0.1 M then 0.2 M potassium phosphate, pH 6.8, 1 mM DTT, at a flow rate of 30 ml/hr and 4 ml fractions were collected. A_{280} (—); GAD activity (---).

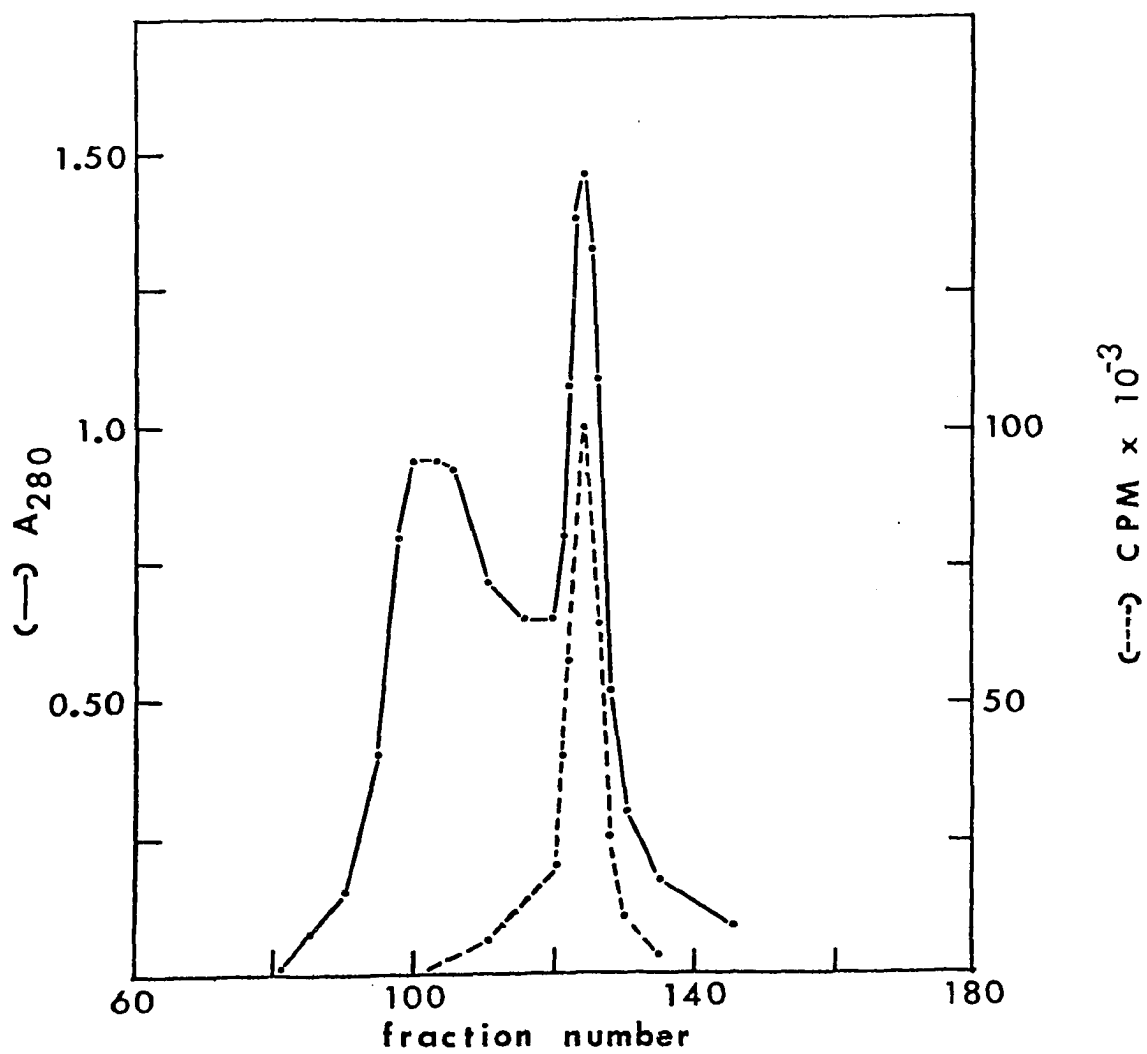


Fig. 4. Elution profile of the Sephadex G-100 column

22.5 ml of the dialyzed pool from the second column was concentrated (PM-30 Diaflow pressure ultrafiltration) to 14.5 ml and was put onto a Sephadex G-100 column (1.5 x 100 cm). The column was eluted with 50 mM potassium phosphate (pH 6.8), 1 mM DTT at a flow rate of 10 ml/hr and 4 ml fractions were collected. A_{280} (—); GAD activity (---).

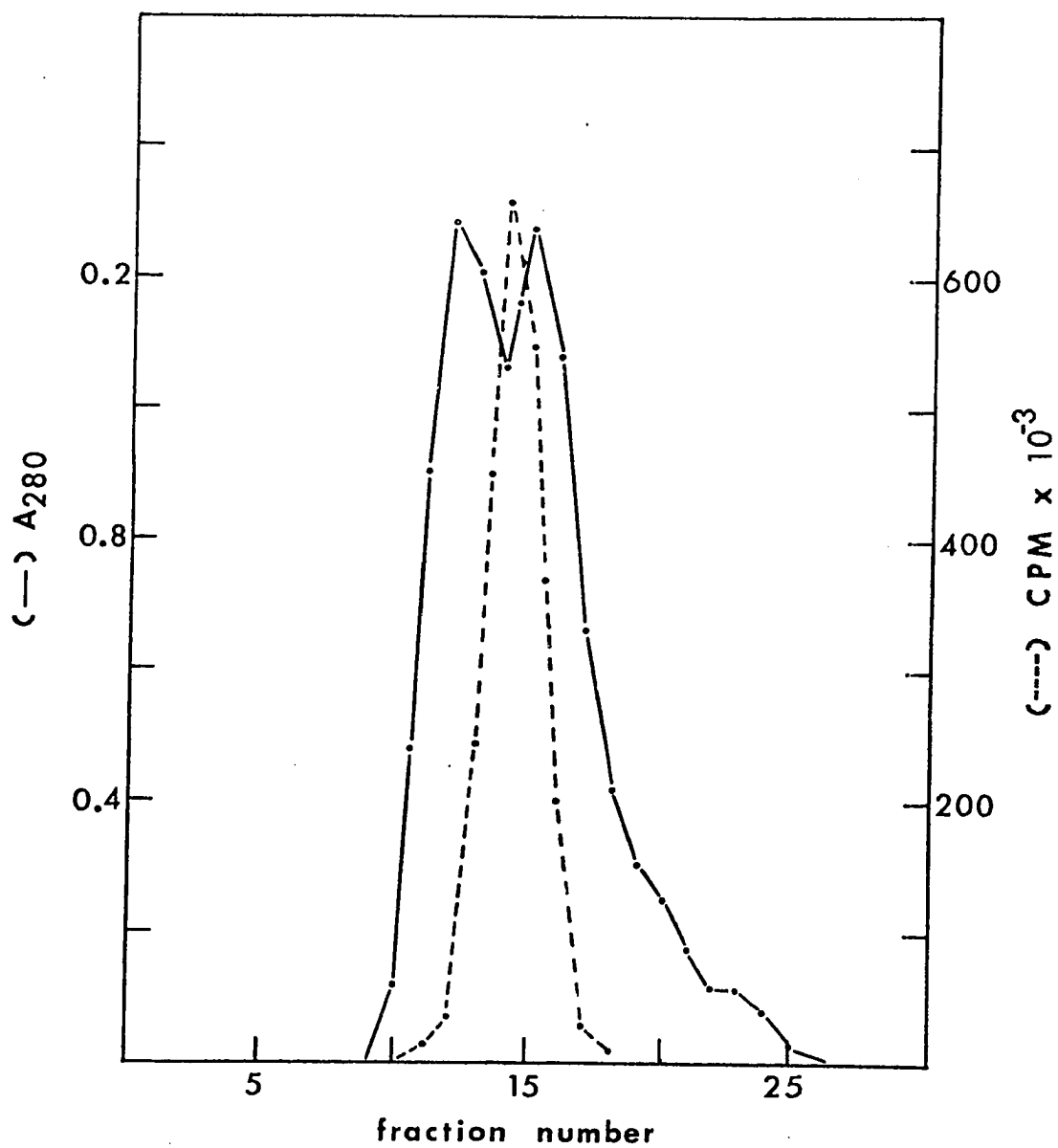


TABLE 1. STEPS IN THE PARTIAL PURIFICATION OF
GLUTAMATE DECARBOXYLASE FROM BOVINE BRAIN

Step	ml	mg/ml	Total mg	CPM/ml*	Total CPM*	CPM/mg*	Yield %	Fold Purification
Crude Extract	227	20.6	4676	2.27	515.3	0.11	100	
Dialyzate	220	11.0	2420	1.87	411.4	0.17	80	1.5
DEAE-Sephadex	65	1.56	101	2.70	175.5	1.74	34	15.8
DEAE-Sephadex	22.5	1.32	29.7	3.00	67.5	2.27	13	20.6
Sephadex G-100	15.5	0.69	10.7	2.42	37.5	3.50	7	32

* 10^{-6}

Further attempts at purification, involving $(\text{NH}_4)_2\text{SO}_4$ fractionation, various phosphate elutions from DEAE-Sephadex columns, preparative disc gel electrophoresis, and further gel filtration on Sephadex G-100 or G-200, uniformly resulted in large losses in GAD activity, and no further increase in specific activity. Reasons for this apparent enzyme instability and methods to cope with this problem are dealt with in the Discussion section.

Isolation and Assay of GAD on Polyacrylamide Gels

The partially purified GAD preparation was analyzed on polyacrylamide disc gel electrophoresis to identify the GAD band. Electrophoresis was performed as described in Methods, the most critical stage being pre-electrophoresis of the separating gel to remove persulfate oxidizing activity. When this was not performed sufficient oxidizing activity was retained in the gel to denature the enzyme and resulted in an inability to detect GAD enzyme activity in gel slices.

The best separation of protein bands was obtained using a 3.5% gel, at pH 9.5. Gels were stained at neutral pH, with minimal surface denaturation by ANS (Hartman and Udenfriend, 1969). Freshly run disc gels were removed from their tubes and immersed in the ANS solution in a Petri dish. The protein bands were then observed under a ultraviolet lamp as yellow fluorescent bands. Gels were then sliced into 2-mm pieces and placed in tubes containing buffer to bring the pH to that necessary for enzyme assay. Gel slices were

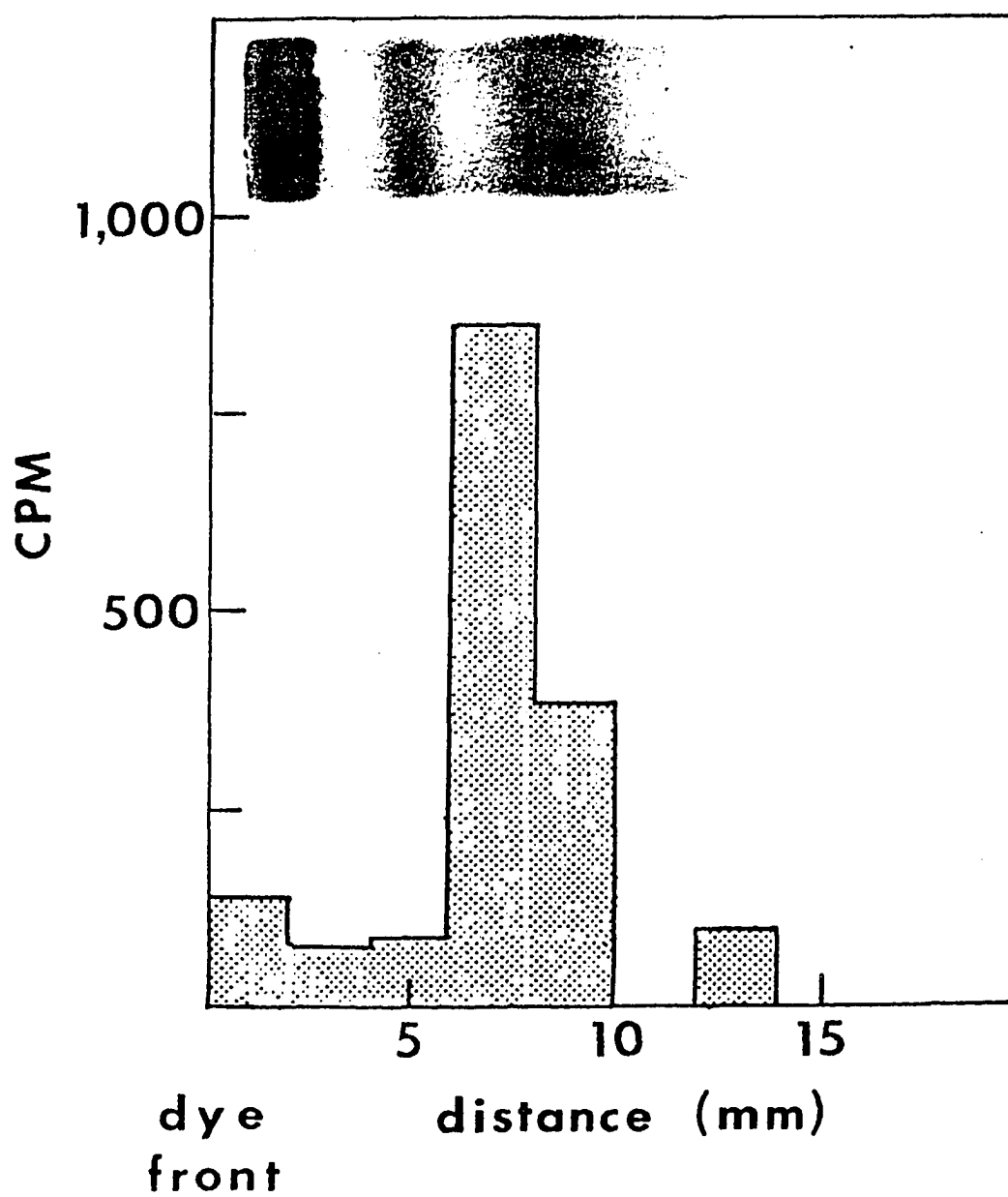
individually assayed as described in Methods.

Figure 5 shows the protein separation obtained with a 3.5% acrylamide gel at pH 9.5. Also the results of assays for GAD activity of that gel are shown. GAD enzyme activity coincides with one major protein band located 7 mm from the dye front. GAD enzyme activity was also determined by slicing individual bands under direct visualization by an ultraviolet lamp. These individual protein bands were then assayed as usual with the detection of enzyme activity confirming the position of the GAD protein.

Although the GAD enzyme protein was detectable as a single band after electrophoresis on 3.5% gels at pH 9.5 this does not mean that this band represents a homogeneous protein. To determine the purity of GAD in that specific band sufficient numbers of standard gels (approximately 50 gels) were run and the GAD bands sliced from each after visualization with ANS and ultraviolet light. These were pooled, homogenized (see Methods) and re-electrophoresed. The re-electrophoresis of a presumably pure protein at multiple pH's and percentages of acrylamide should demonstrate the homogeneity of the sample if in fact it is a single protein. Since the basis of protein separation in disc gel electrophoresis is by molecular weight and charge, it is possible that two proteins of differing charge may migrate to the same staining position on disc gels if the proteins have compensating molecular weight characteristics. Thus, the re-electrophoresis of the GAD band under varying pH's

Fig. 5. Disc electrophoresis of partially purified GAD in acrylamide gels at pH 9.5 and the assay for GAD enzyme activity of that gel

The protein pattern is on the top of the figure. A 100 μ g protein fraction from the Sephadex G-100 column was loaded onto a 3.5% acrylamide, pH 9.5 gel. The gel is shown with the origin to the right, the dye front to the left. Full details for electrophoresis and GAD enzyme assay are given in Methods.



and gel percentages would indicate if contaminating proteins were present.

The re-electrophoresis described above was performed in 3.5, 5.25, 7.0, and 10.5 percent acrylamide gels at pH 9.5. Figure 6 shows the re-electrophoresis of the GAD band at multiple percentages of acrylamide. One major protein which coincided with GAD activity was found at all acrylamide percentages with extremely minor contaminants visible in the 10.5% gel in the immediate vicinity of the GAD enzyme protein. Staining of the 5.25% gel with ANS and slicing of the band under ultraviolet lamp illumination, followed by enzymatic assay of the gel (Fig. 6), confirms that the original protein band (Fig. 5) at 7 mm on the 3.5%, pH 9.5 gel is indeed the GAD enzyme protein. Figure 7 shows the re-electrophoresis of the GAD enzyme protein in a 7% gel in two different anodic gel systems, pH 10 and pH 9.5. In each case only one major band is present thus supporting the previous results on the homogeneity of the GAD protein as isolated on disc gel electrophoresis. Figure 8 shows the re-electrophoresis of the GAD enzyme protein at multiple percentages of acrylamide in the pH 10 system which further supports the above conclusion.

Immunochemical Analysis

With the homogeneity of the electrophoretically obtained GAD protein established a large scale isolation procedure for the enzyme was carried out. Preparations from the G-100 column were run in sufficient numbers on 3.5% pH 9.5 gels

Fig. 6. Re-electrophoresis of the GAD band at multiple percentages of acrylamide at pH 9.5 and the enzyme assay of those gels

The protein patterns are on the top of the figure. The gels are shown with the origin to the right, the dye front to the left. Full details for re-electrophoresis and GAD enzyme assay are given in Methods. From top to bottom the samples are: 3.5%, 5.25%, 7.0%, 10.5%. Enzyme assay was done on the 5.25% gel.

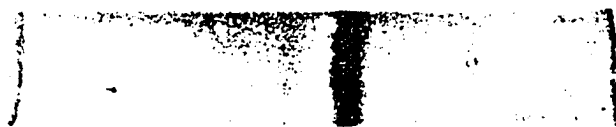
3.5%



5.25%



7%



10.5%

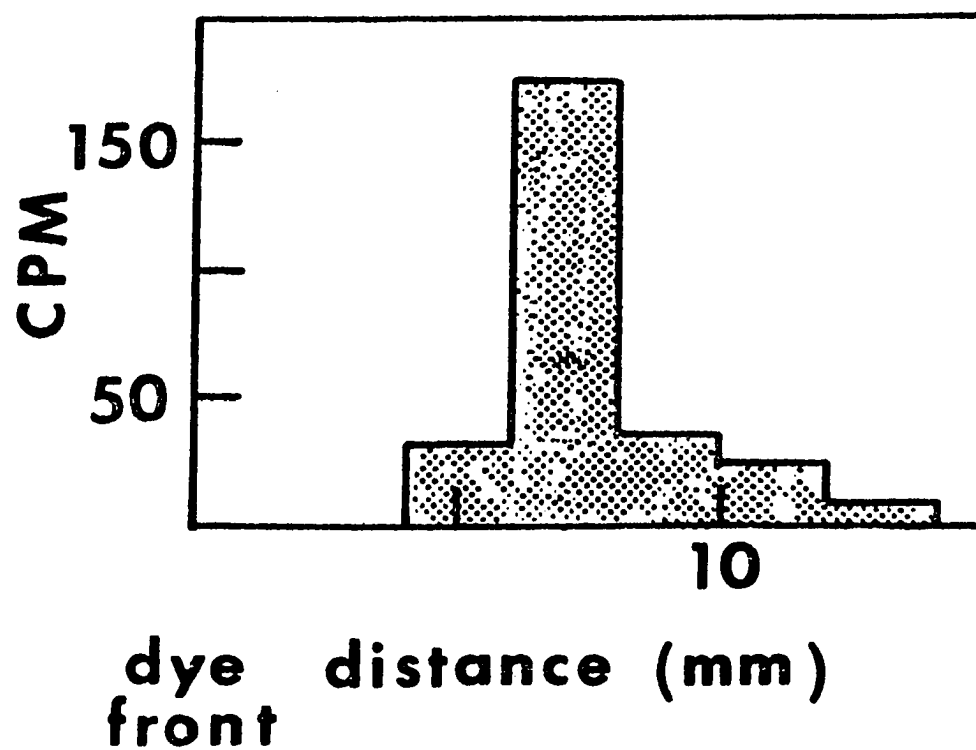
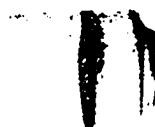


Fig. 7. Re-electrophoresis of the GAD band in 7% gels in the pH 10 and pH 9.5 anodic systems

The gels are shown with the origin to the top, the dye front to the bottom. Full details for re-electrophoresis are given in Methods. Left gel is pH 10; right gel is pH 9.5.

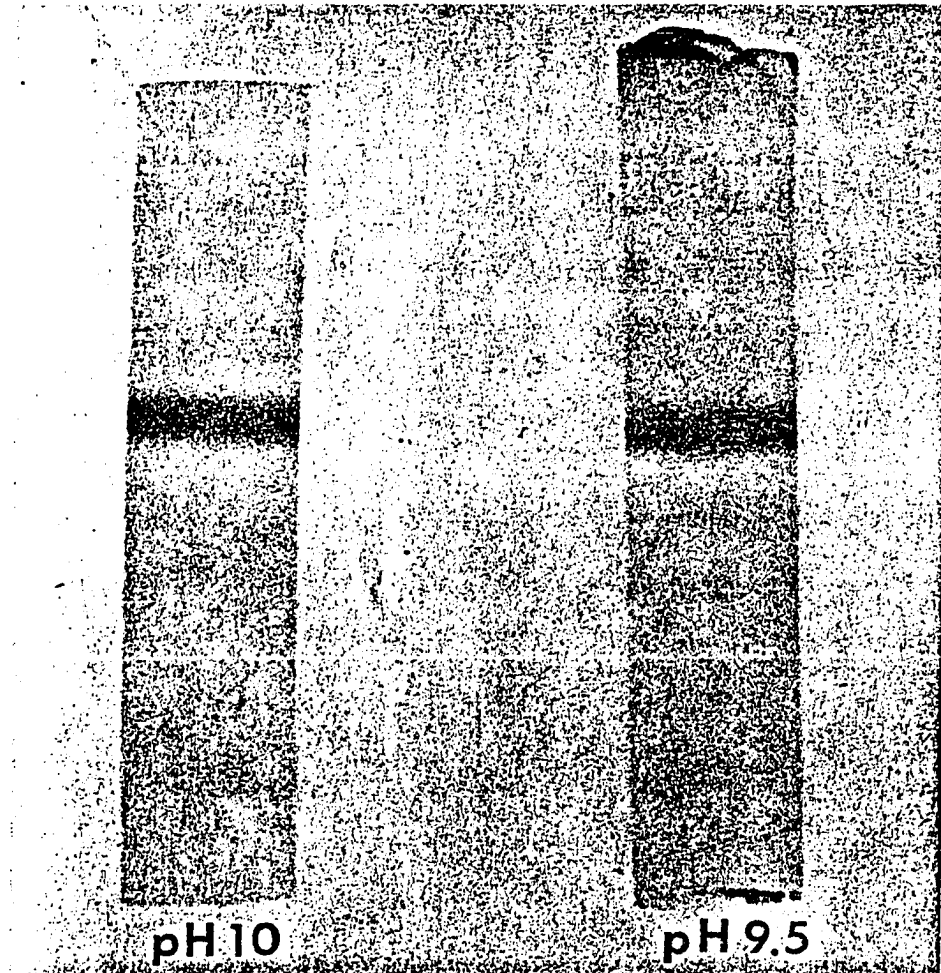
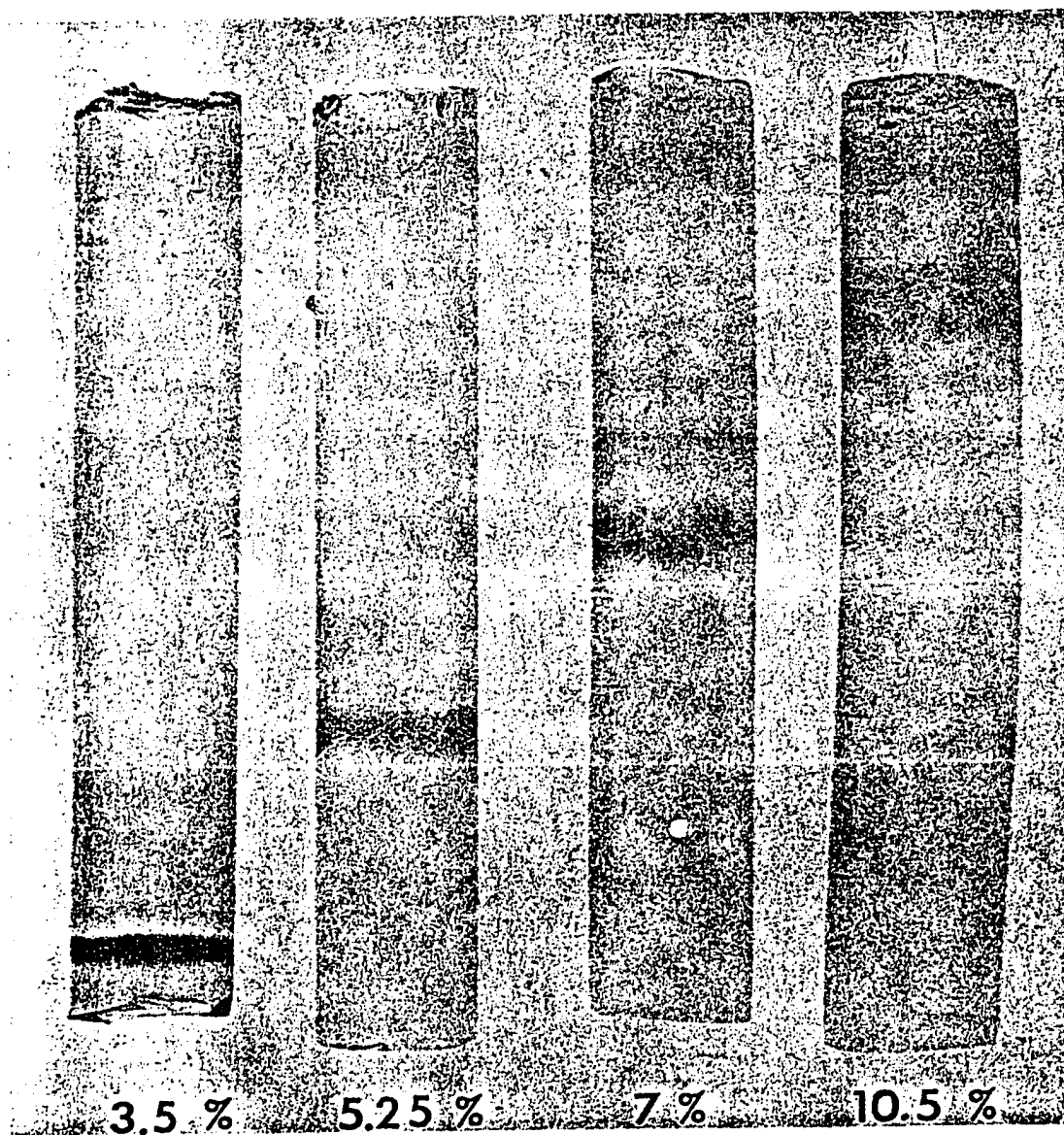


Fig. 8. Re-electrophoresis of the GAD band at multiple percentages of acrylamide at pH 10

The gels are shown with the origin to the top, the dye front to the bottom. Full details for re-electrophoresis are given in Methods. From left to right the samples are: 3.5%, 5.25%, 7.0%, 10.5% acrylamide.



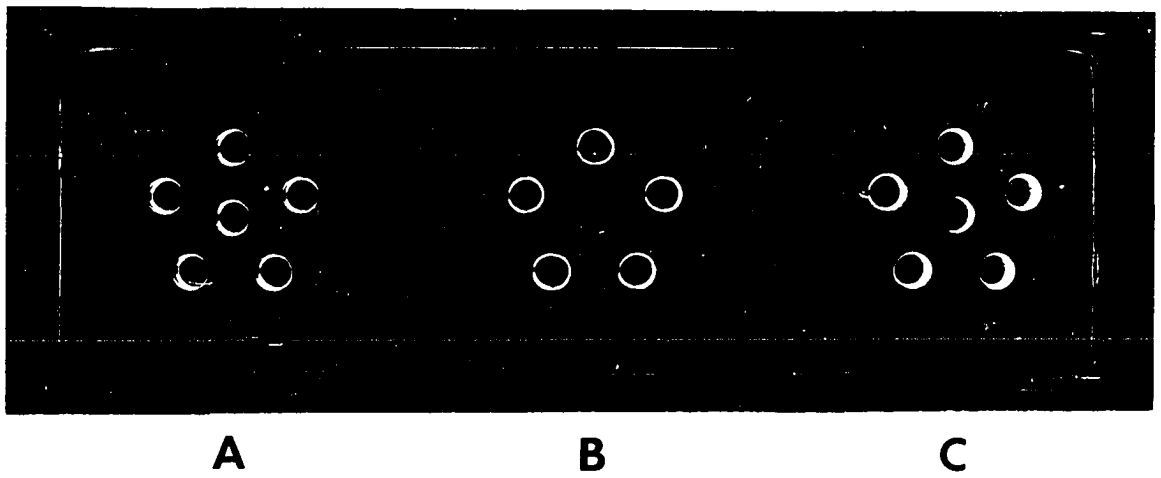
so that 250 μ g of pure enzyme per inoculating dose could be prepared. This required the electrophoresis of approximately 850 standard gels to isolate large enough quantities of GAD protein for injection. The injection protocol is described in the Methods section. Antisera from rabbits receiving a total of 850 μ g of purified bovine GAD were used in all subsequent experiments.

To determine if the anti-GAD sera were monospecific micro double immunodiffusion plates were employed. The antigens used were the partially purified bovine GAD, bovine brain homogenate, and mouse brain homogenate. Figure 9 shows the results of this experiment. This plate was refrigerated overnight and washed in 0.9% NaCl to clear the non-precipitating proteins. The antisera to GAD isolated from bovine cerebellum were found to react with the decarboxylase from mouse brain with a reaction of identity. A sharp precipitin band was observed with the partially purified bovine GAD as an antigen (Figure 9A). The anti-GAD sera also gave a single sharp precipitin line with the bovine brain (Figure 9B) and mouse brain (Figure 9C) homogenates. Sera from unimmunized rabbits gave no precipitin band on a micro double immunodiffusion plate. The results shown in Figure 9B and 9C demonstrate that the anti-GAD antisera were monospecific for GAD and did not contain detectable quantities of precipitating antibodies to any other proteins found in brain homogenates.

Prior to further comparisons between bovine and mouse

Fig. 9. Monospecificity of rabbit anti-bovine GAD sera on a micro immunodiffusion plate

Anti-GAD sera were obtained as described in Methods. The center wells contain anti-bovine GAD sera. Antigen is in outer wells. A: partially purified bovine GAD adjusted to 1.45 mg protein/ml; B: bovine cerebellum homogenate adjusted to 7.0 mg protein/ml; C: mouse brain homogenate adjusted to 5.0 mg protein/ml.



glutamic acid decarboxylase an experiment was conducted to determine if GAD enzyme activity was linear with respect to the quantity of mouse brain homogenate assayed. Figure 10 indicates that the assay used for a bovine homogenate also gave a valid estimation of GAD enzyme activity in a mouse brain homogenate. The assay was highly reproducible from one preparation to the next.

Thermal Stability

Experiments were performed to study the stability of the enzyme with time at various incubation temperatures. The stability of GAD at 4° C, 30° C, 40° C, and 50° C from bovine brain, 4 day old mouse brain, and 20 day old mouse brain were determined. Different ages of mouse brain were chosen to determine if there was a difference between GAD stability in the young and older mice. Figure 11 shows the results of this experiment. For all brain extracts incubated at 50° C enzyme activity is reduced to less than 10% of initial activity by 1 hour. At incubation temperatures of 30° C and 40° C the bovine brain GAD appeared to decrease less in enzymatic activity than the mouse preparations. After a 4 hour incubation period at 30° C, 60% of the bovine GAD activity remained while at 40° C, 52% of the activity was retained. At the same time periods, the mouse preparations retained only between 35-50% initial activity at 30° C, and 8-20% initial activity at 40° C. Results of thermal stability at 4° C were similar for all brain extracts, ranging between 75-93% of initial activity being retained.

Fig. 10. GAD enzyme activity with respect to protein
concentration of a mouse brain extract

The mouse brain homogenate was prepared as described
in Methods.

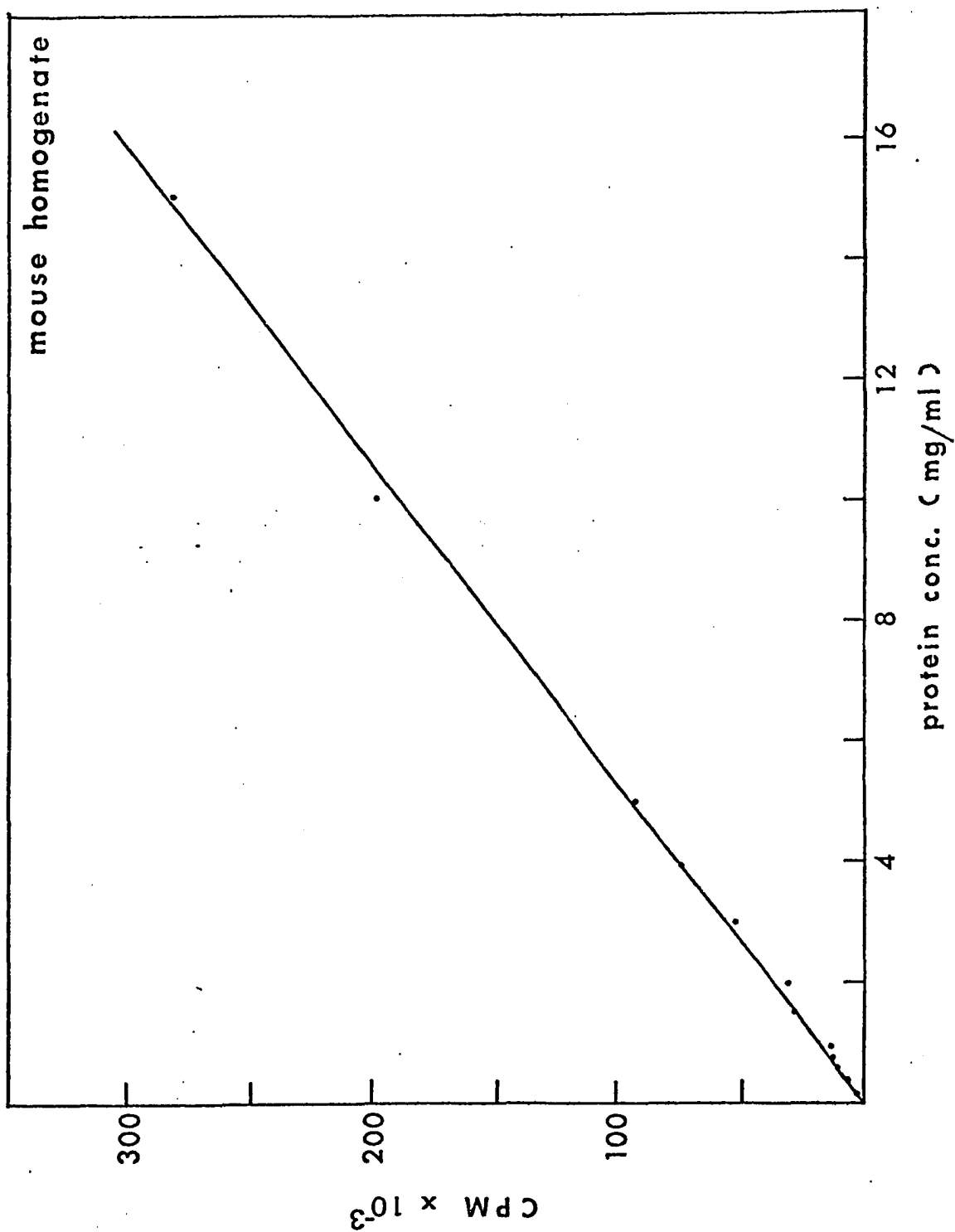
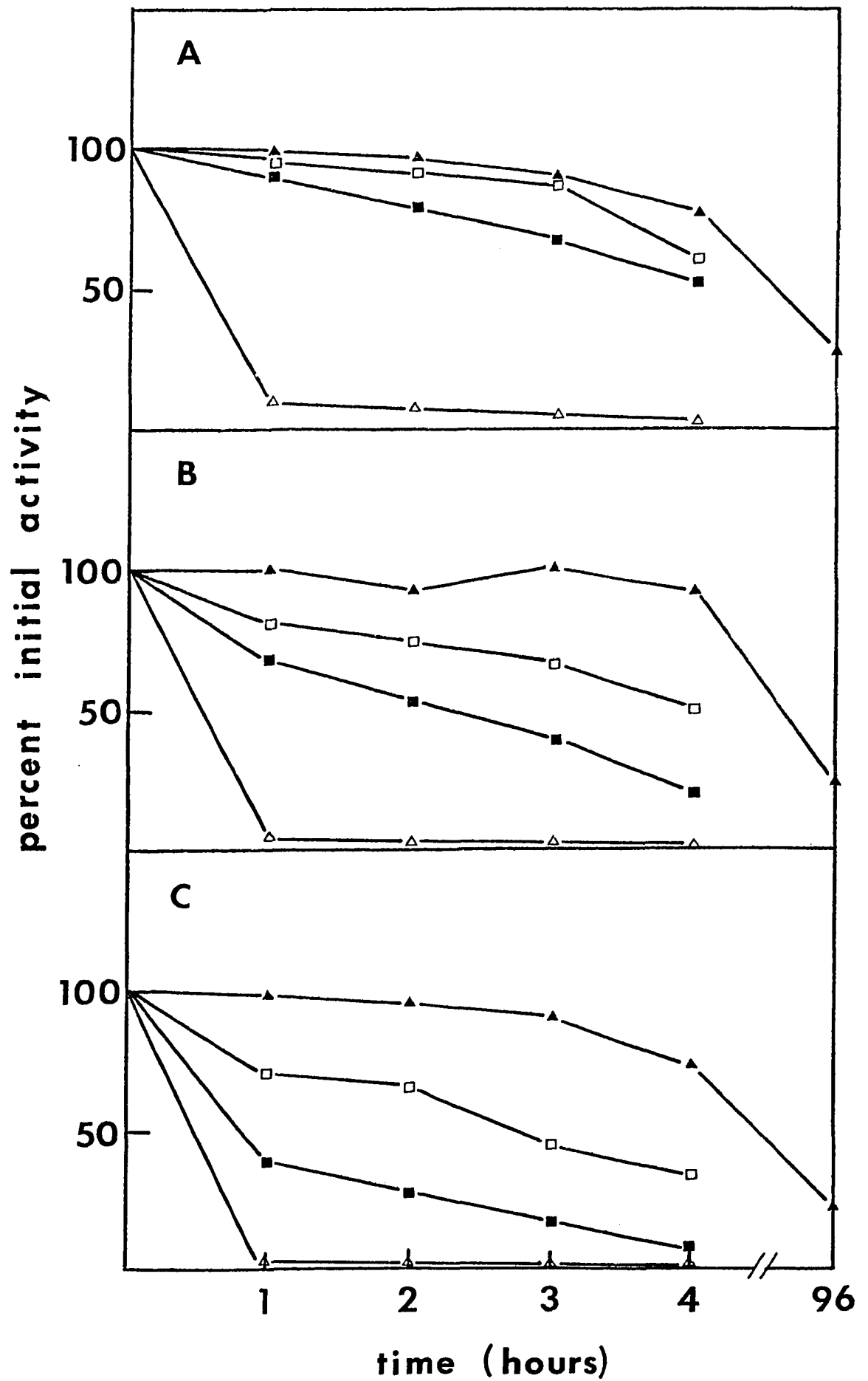


Fig. 11. Thermal stability of a bovine and mouse brain
GAD

Brain preparations were adjusted to 4 mg protein per ml and incubated at various temperatures. Percent initial GAD enzyme activity is shown as a function of time at 4° C (▲), 30° C (□), 40° C (■), 50° C (△). A: bovine cerebellum; B: 4 day old mouse brain; C: 20 day old mouse brain.



pH Optimum

The activity of GAD at various pH's was studied using brain preparations from bovine cerebellum, 4 day old mouse, and 20 day old mouse. Aliquots of brain preparations were adjusted to pH values between 5.7-7.9 and assayed for GAD activity. The results (Figure 12) show the optimal pH for GAD enzyme activity was 6.8 for all preparations.

Effects of Potassium on GAD Activity

The effect on mouse brain GAD activity of the addition of potassium chloride or potassium phosphate to 50 mM potassium phosphate buffer was studied for a 18 day old mouse brain. The results of the experiment (Figure 13) show that KCl added to 50 mM potassium phosphate is much more inhibitory than equimolar amounts of potassium phosphate. Similar results were obtained with NaCl showing that inhibition could be attributable to Cl^- ion.

Inhibition of GAD by Anions

The inhibition of mouse brain GAD enzyme activity by the addition of various concentrations of potassium salts was examined. The following compounds were evaluated for their inhibitory effects at concentrations between 0.05-0.35 M: potassium acetate, potassium chloride, potassium nitrate, potassium sulfate, potassium iodide, and potassium fluoride. The enzyme was pre-incubated with inhibitor at room-temperature for 1 hour prior to the addition of the substrate. The results (Figure 14) of this experiment show that at low con-

Fig. 12. The effect of pH on bovine and mouse brain GAD

Brain homogenates were adjusted to 4 mg protein per ml. A: bovine cerebellum; B: 4 day old mouse brain; C: 20 day old mouse brain.

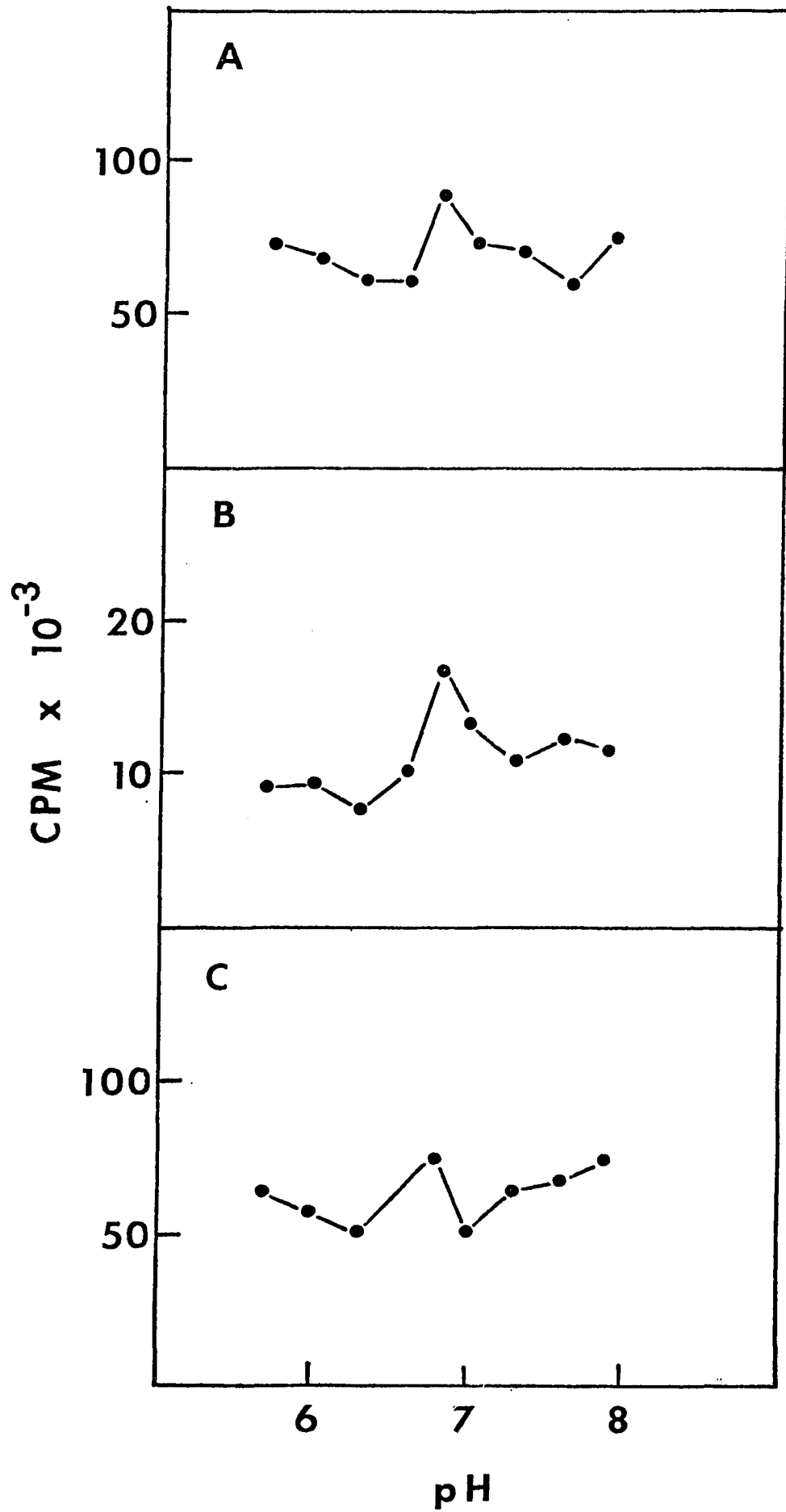


Fig. 13. Effect of potassium on mouse brain GAD activity

18 day old mouse brain extracts were adjusted to 4 mg protein/ml. Percent initial GAD enzyme activity is shown as a function of K-phosphate (•) molarity, KCl (■), and NaCl (o).

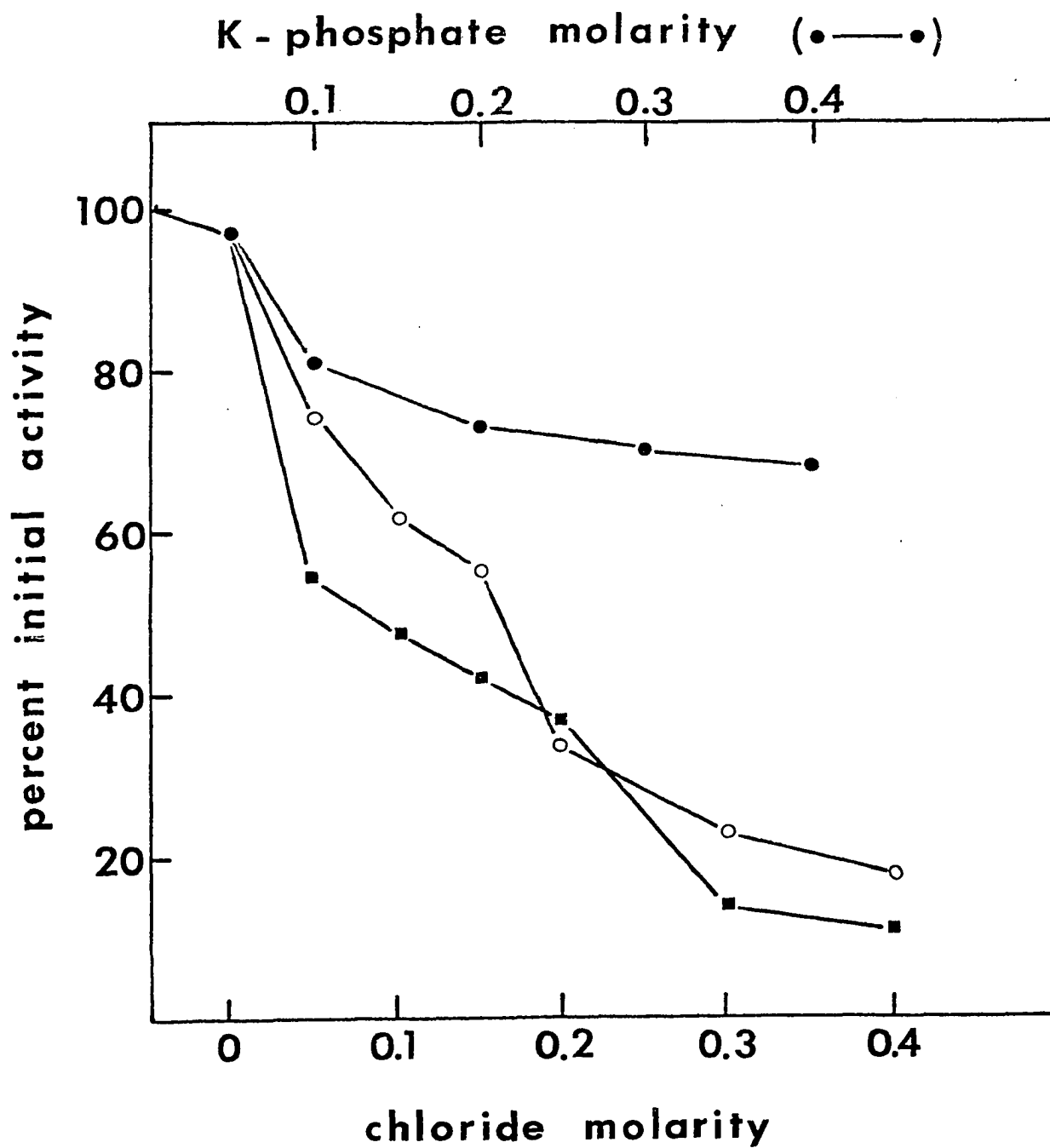
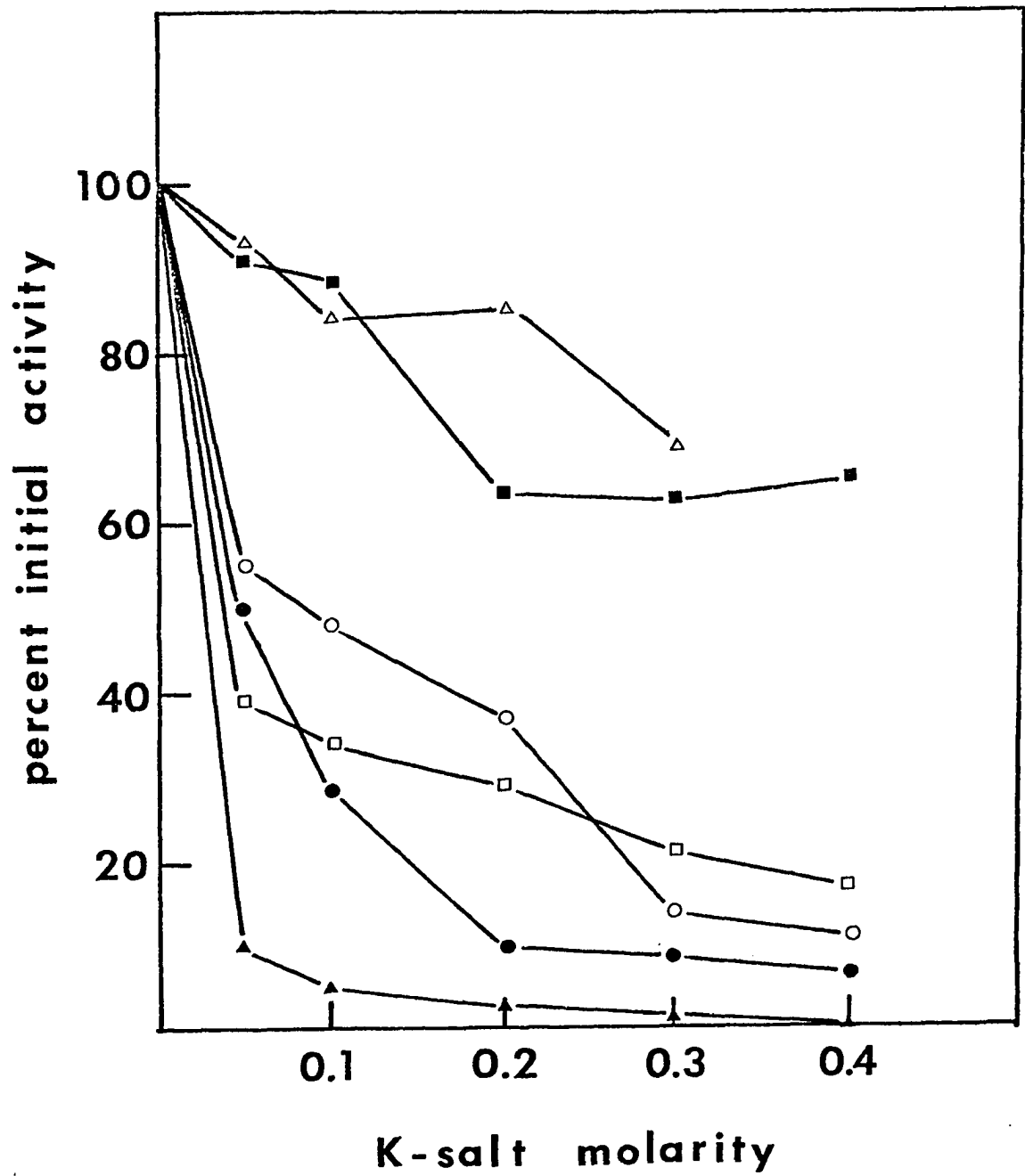


Fig. 14. Inhibition of mouse brain GAD enzyme activity by the addition of various concentrations of potassium salts

18 day old mouse brain extracts were adjusted to 4 mg protein/ml. Percent initial GAD enzyme activity is shown as a function of K-salt molarity for: KF (Δ), $\text{KC}_2\text{H}_3\text{O}_2$ (\blacksquare), KCl (\circ), KNO_3 (\bullet), K_2SO_4 (\square), KI (\blacktriangle).



centrations KI is the most potent inhibitor followed by K_2SO_4 , KNO_3 , KCl , $KC_2H_3O_2$, and KF . At concentrations of KNO_3 and KCl above 0.05 M the inhibition of GAD increases while K_2SO_4 reaches a maximum of inhibition at 0.4 M.

Table 2 summarizes the data of Figure 14 including a comparison of inhibition by those compounds of bovine cerebellum, 19 day old mouse brain, pig, sheep, and human brain GAD. Inhibition of GAD activity was examined at a 0.1 M concentration of all compounds. There is approximately equal inhibition of the various brains' GAD enzyme activity by each of the compounds studied.

Inhibition of GAD by Various Compounds

An initial experiment was performed to determine the extent of inhibition of GAD by various compounds between the concentrations of 0.001-1.0 mM. The following compounds were examined at an inhibitor concentration of 0.3 mM for their effect on mouse and bovine GAD enzyme activity: hydroxylamine, iodoacetamide, 1,2-napthoquinone-4-sulfonic acid, nitroso R salt, aminooxyacetic acid, and p-hydroxy-mercuribenzoic acid. As in the previous study the enzymes were pre-incubated at room-temperature for one hour in the presence of the inhibitor compound. Table 3 indicates the results of this experiment. 1,2-Napthoquinone-4-sulfonic acid is the most potent inhibitor of both glutamic acid decarboxylases, whereas iodoacetamide and hydroxylamine are the weakest inhibitors. In each of these cases the inhibitors affect bovine and mouse glutamic acid decarboxylase

TABLE 2. COMPARISON OF INHIBITION BY POTASSIUM SALTS OF
BOVINE CEREBELLUM, 19 DAY OLD MOUSE BRAIN, PIG,
SHEEP, AND HUMAN BRAIN GAD ENZYME ACTIVITY

Compound*	<u>Inhibition %</u>				
	Mouse	Bovine	Pig	Sheep	Human
Pot. Phos.	19	26	14	11	+9
KCl	52	52	45	24	42
NaCl	38	51	36	44	36
KI	92	94	87	90	72
KF	47	0	4	21	35
KC ₂ H ₃ O ₂	17	18	+17	29	28
K ₂ SO ₄	70	68	55	70	73
KNO ₃	71	70	59	57	46

*All values are for 0.1 M concentration of the salt.

100% activity is that in 0.05 M potassium phosphate buffer.

TABLE 3. COMPARISON OF INHIBITION BY VARIOUS
COMPOUNDS OF BOVINE CEREBELLUM AND 19
DAY OLD MOUSE BRAIN GAD ENZYME ACTIVITY

Compound (0.3 mM)	<u>Inhibition %</u>	
	Mouse	Bovine
Hydroxylamine	12	11
Iodoacetamide	18	11
1,2-Napthoquinone-4-sulfonic acid	100	97
Nitroso R salt	65	94
Aminooxyacetic acid	62	78
p-Hydroxymercuribenzoic acid	59	84

activity to a similar extent. Although differences in inhibition of the two mammalian glutamic decarboxylases may be noted for aminooxyacetic acid, nitroso R salt, and p-hydroxymercuribenzoic acid, each of these compounds appear to be more potent inhibitors of the bovine GAD than of the mouse GAD.

Effect of Pyridoxal-5'-Phosphate on GAD Activity

GAD activity was studied as a function of pyridoxal-5'-phosphate concentration (Figure 15). All assays systems were pre-incubated for 2 hours at room-temperature to allow interaction of pyridoxal-5'-phosphate with GAD. For all three preparations maximal GAD enzyme activity was exhibited at the same pyridoxal-5'-phosphate concentration. Maximal activity of GAD enzyme activity occurred at a 0.025 mM pyridoxal-5'-phosphate concentration for both the 4 and 28 day old mouse GAD. A plateau of maximal activity of GAD exists between a 0.010-0.025 mM concentration for the bovine cerebellum GAD. On either side of the optimum concentration of pyridoxal-5'-phosphate GAD enzyme activity rapidly decreased.

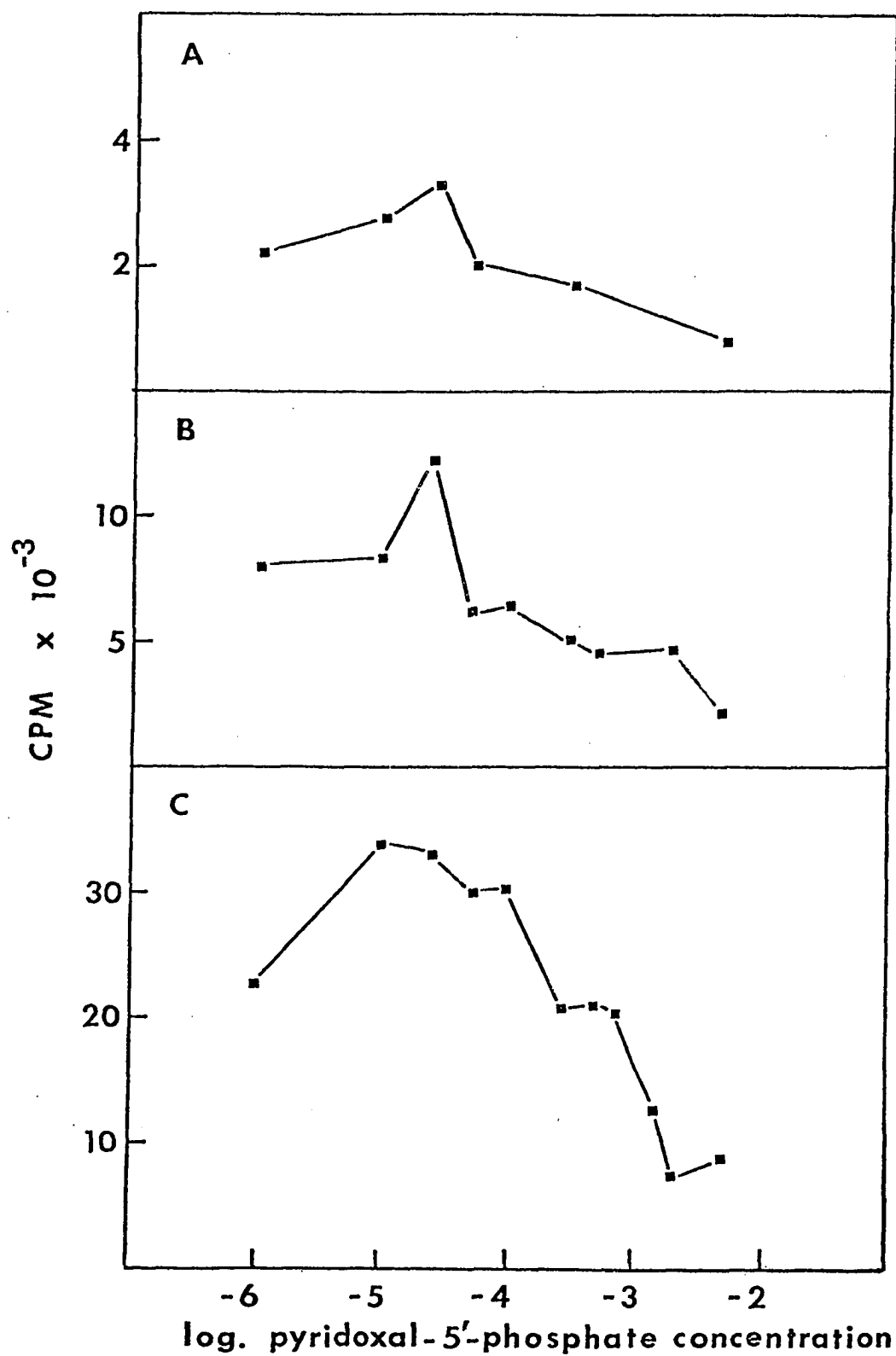
Glutamate Decarboxylase in Developing Mouse Brain

GAD specific activity was examined at various postnatal ages of developing mouse brain between 1 and 90 days. Mice (C57BL/6J) were maintained as described in Methods. A minimum of three brains were used at any age point. Brains were homogenized (Duall homogenizer, Kontes Glass Co.) in

Fig. 15. Effect of pyridoxal-5'-phosphate concentration on GAD activity

All enzyme preparations were adjusted to 4 mg protein per ml. GAD activity is shown as a function of the log of pyridoxal-5'-phosphate concentration between 0.001-4 mM.

A: 4 day old mouse brain; B: 28 day old mouse brain; C: bovine cerebellum.



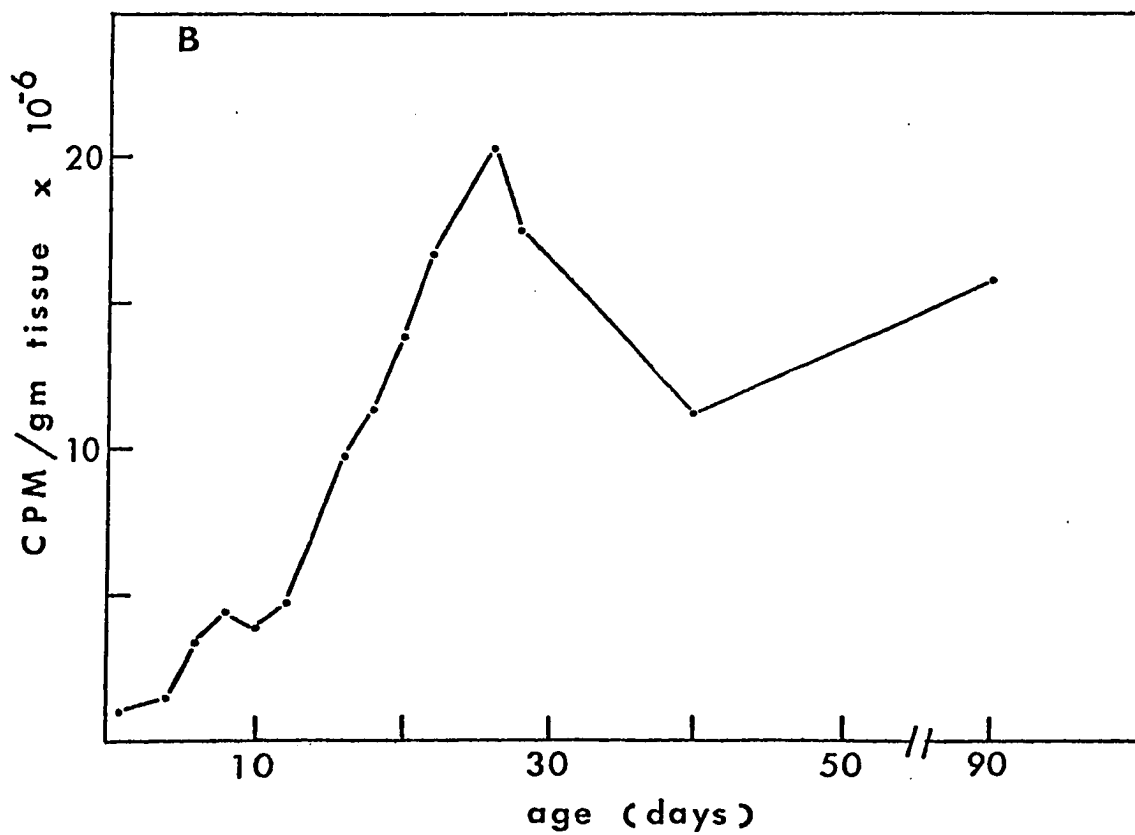
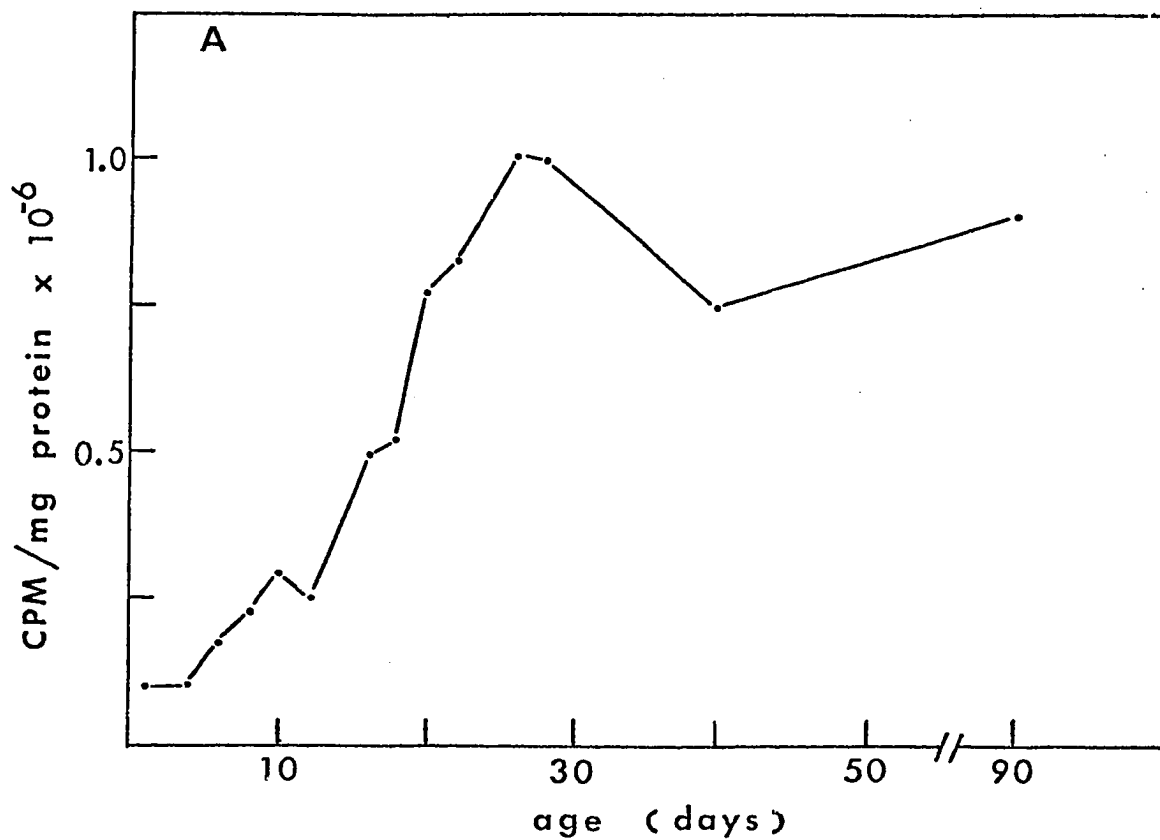
2.5 volumes of potassium phosphate buffer A, centrifuged at 35,000 x g for 20 min at 4° C and the post-mitochondrial supernatant fraction retained. This fraction was assayed for GAD activity.

Figure 16 shows the results of this experiment expressing units of GAD specific activity in two alternative forms: Figure 16A expresses units of enzyme specific activity as CPM in CO₂ released from L-(1-¹⁴C)glutamic acid per mg protein; Figure 16B contains the enzyme activity expressed as CPM in CO₂ released from L-(1-¹⁴C)glutamic acid per gram wet weight tissue. Both show an initial rise in specific activity occurring at 6 days postnatally followed by a rapid increase in enzymatic activity which reaches a maximum at 28 days postnatally. GAD specific activity decreases between 28-40 days, then the level of enzyme activity shows a slight rise for the period of time between 40-90 days postnatally. Considering both pieces of data, GAD enzyme specific activity appears to increase 12-15 fold between 6 and 28 days in mouse brain development then decreases to an activity that is approximately 10 fold higher than the initial activity of enzyme found at one day after birth.

Quantitative immunoprecipitation determinations of mouse GAD using rabbit anti-bovine GAD antisera were performed at various ages of developing brain as described in Methods. However, before this study was undertaken a preliminary experiment was designed to evaluate the ability of anti-GAD antisera to inhibit GAD. It is of importance

Fig. 16. GAD activity during postnatal development in mouse brain

Mouse brain extracts were prepared as described in text. Three mice (C57BL/6J) were used at any age point. A: CPM in CO_2 released from L-(1- ^{14}C)glutamic acid per mg protein; B: CPM in CO_2 released from L-(1- ^{14}C)glutamic acid per gram wet weight tissue.



to determine if a relationship exists between inhibition of GAD enzyme activity and increasing concentrations of anti-GAD sera prior to using the antiserum for enzyme protein quantitation. The results (Figure 17) indicate that the inhibition of enzyme activity by sera is not a totally linear effect. A maximum of 90% inhibition of partially purified bovine cerebellum GAD (Figure 17A) and of a mouse brain GAD (Figure 17B) was obtained. Increased concentrations of anti-GAD sera did not result in further inhibition of the enzyme. Therefore, calculations for quantitative immunoprecipitation data were made at dilutions of anti-GAD sera where the inhibition of GAD activity was linear with respect to immune sera concentration.

The results of the experiment on the quantitative immunoprecipitation of GAD in developing mouse brain are shown in Figure 18. The amount of protein in the immune precipitate per brain at each age point increases 10 fold over the period between 1-28 days postnatally, then decreases to approximately 7 fold of initial GAD enzyme protein levels at more mature ages. The volume of anti-GAD antisera required for complete inhibition of all GAD enzyme activity per brain at each age point increases to 9.5 times initial quantities by 22 days postnatally. Between 22-90 days the quantity of sera required for total GAD inhibition decreases to 7 fold then slowly increases to 11 times initial quantities.

The superimposition of the GAD enzyme activity profile

Fig. 17. Inhibition of partially purified bovine GAD and mouse GAD enzyme activity by rabbit anti-bovine GAD sera

Inhibition of GAD activity by anti-GAD sera was performed as described in Methods. GAD activity (\circ) in the supernatant after immunoprecipitation; percent inhibition of initial GAD activity (\bullet). A: partially purified bovine cerebellum GAD; B: mouse brain homogenate.

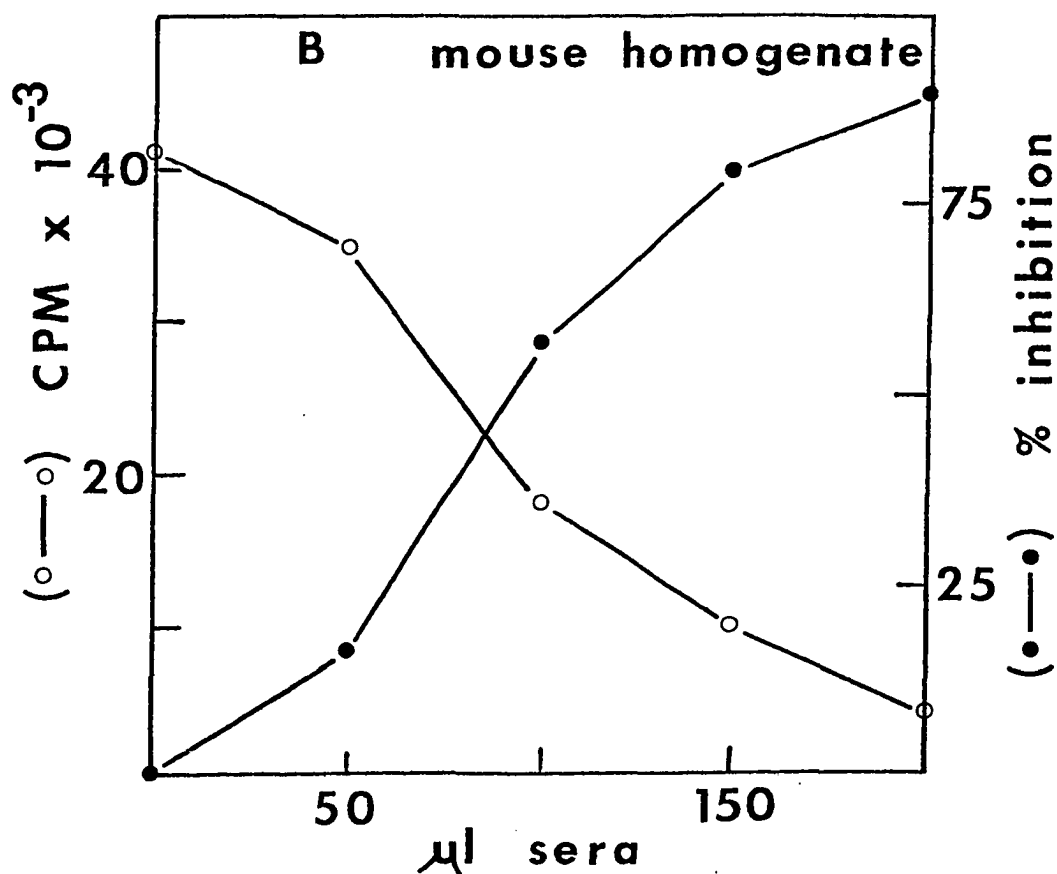
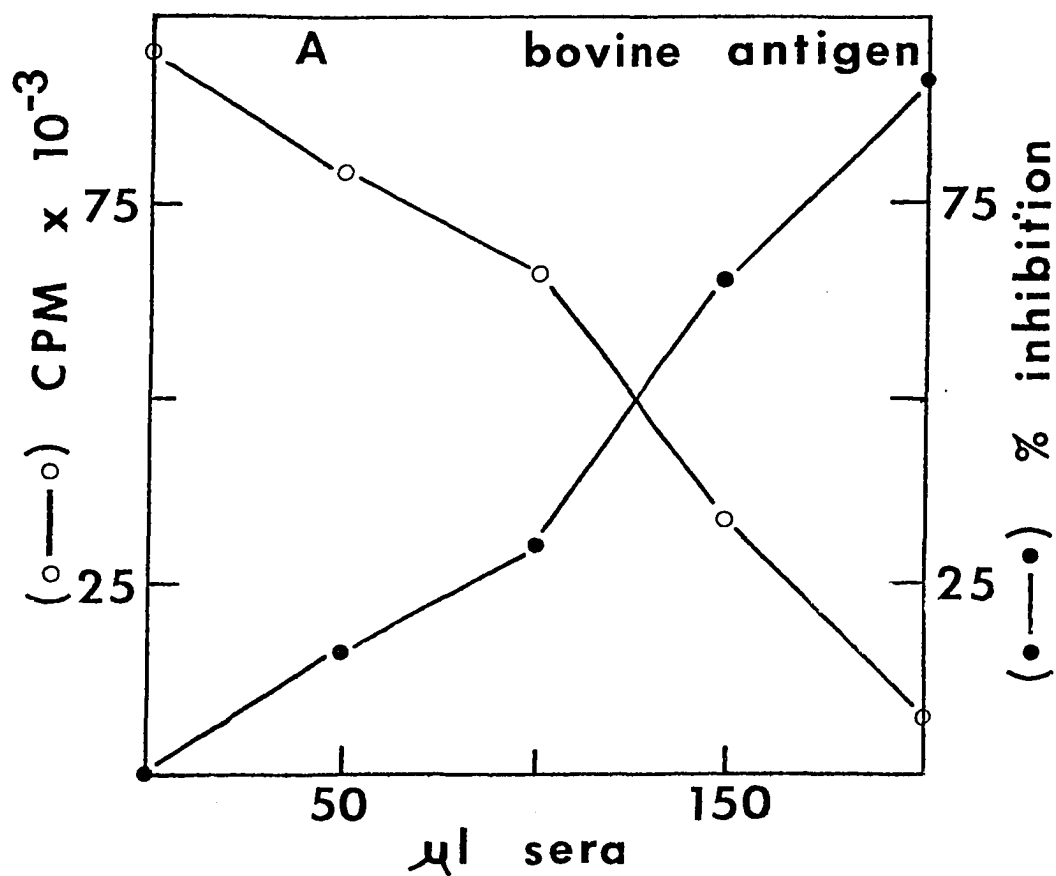
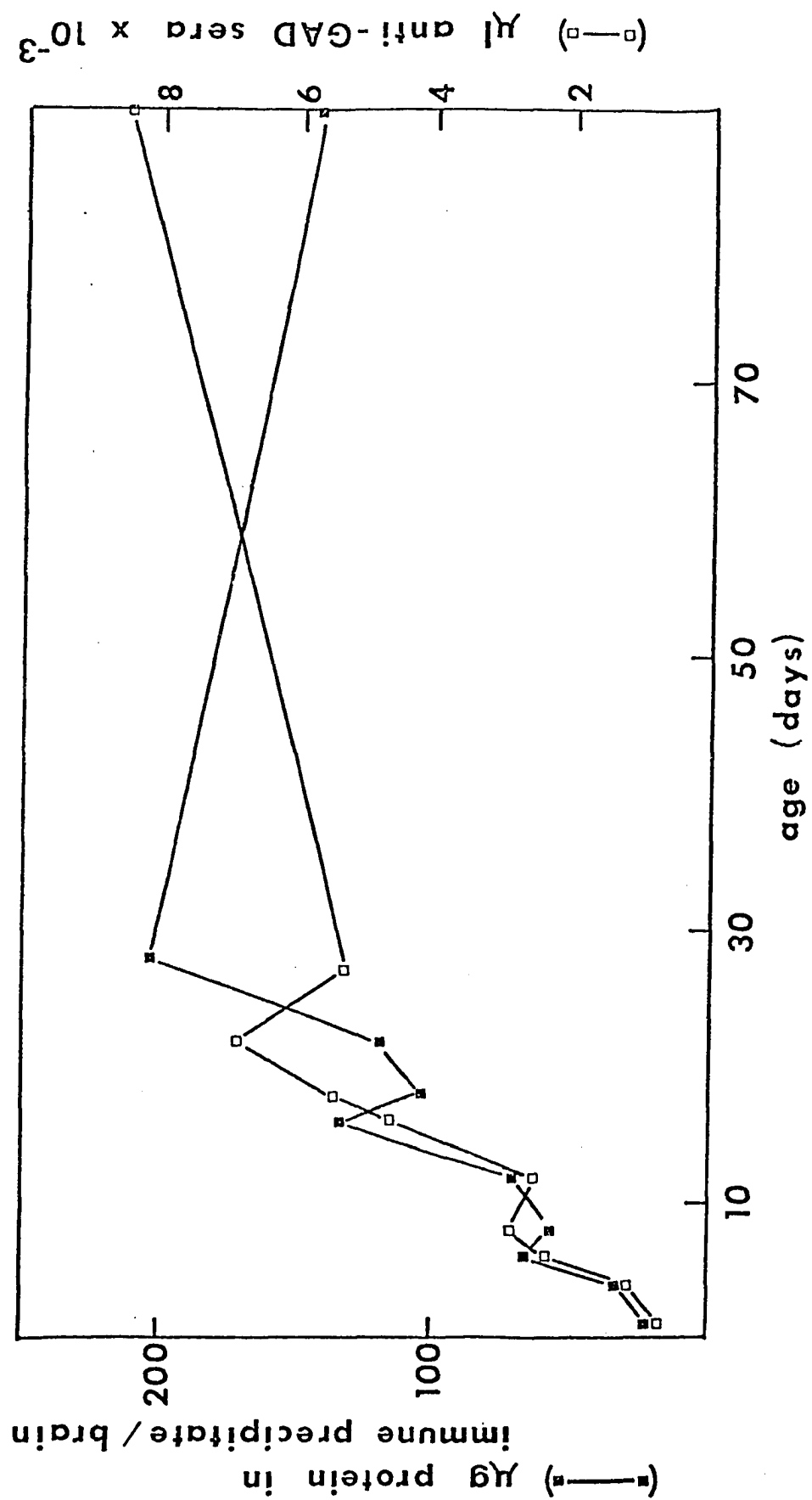


Fig. 18. Quantitative immunoprecipitation of GAD in developing mouse brain

Quantitative immunoprecipitations were performed as described in Methods. Micrograms of protein in the immune precipitate per brain (■); microliters of anti-GAD antisera required for complete inhibition of all GAD enzyme activity per brain (□).



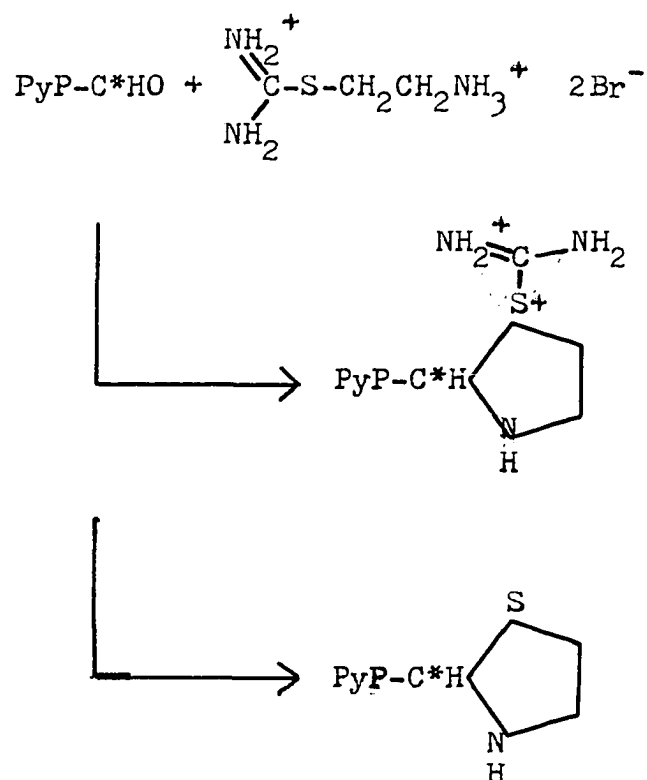
(Figure 16) onto the data just presented (Figure 18) indicate that both profiles closely coincide. Both indicate that initial rises in GAD occur at day 6 with maximal levels reached between 22-28 days postnatally. "Mature" levels of the enzyme appear to exist between 28-90 days.

DISCUSSION

Studies with a crude mouse brain extract have shown that GAD is a sulfhydryl enzyme and requires PyP for enzyme activity (Roberts and Frankel, 1951; Roberts and Simonsen, 1963). In 1966 Susz, Haber, and Roberts were able to accomplish a partial purification of GAD from mouse brain by the use of a combination of PyP and 2-aminoethyl-isothiouronium bromide (AET) which stabilized the enzyme for long enough periods to allow a purification to be performed.

It had previously been discovered that pyridoxal-5'-phosphate requiring enzymes, in general, exhibit an inactivation by U.V. light or sunlight (Schlenk *et. al.*, 1946), and that a soluble preparation of mouse brain GAD at 4° C in the presence of glutathione (GSH) and PyP was inactivated more rapidly in the light than in the dark. Roberts (1972) proposed that the apoenzyme of GAD, as well as the coenzyme, may be inactivated by light-induced free radicals since the enzyme failed to be reactivated by an excess of PyP. It was found that a combination of GSH and PyP, both of which are known to react with free radicals, provided partial protection of GAD against U.V. inactivation. The examination of other radioprotective substances resulted in the choice of AET; AET had previously been reported to exist in neutral solution as 2-mercaptoethylguanidine (Khyme *et. al.*, 1957). AET protected GAD somewhat better from U.V. inactivation than isomolar amounts of GSH or dithioerythritol (DTE).

Spectral data (Buell and Hansen, 1960) indicates that AET may form a cyclic derivative with PyP, or that AET is merely a source of mercaptoethylamine, either of which then forms a thiazolidine derivative with the aldehyde moiety of PyP.



From inhibition data Roberts (1964) postulated the existence of a thiazolidine bridge in GAD. The sensitivity to destruction of the thiazolidine bridge might be expected to be the same as that of the active site on GAD. Thus, the probability of the active site being destroyed by light-induced free radicals might be less in the presence of the thiazolidine complex than in the presence of isomolar concentrations of

GSH and PyP. This theory might explain the greater in vitro protection of GAD by AET when compared with sulfhydryl compounds lacking amino groups.

Although previous work (Roberts, 1972) indicates that aminothiods and AET in particular are superior GAD-protecting agents as compared to sulfhydryl compounds, it was found that for the experiments described in this work dithiothreitol (DTT) provided the greater protection against enzyme inactivation. DTT was found to offer a greater stabilization of GAD than 2-mercaptoethanol, DTE, or AET.

The partial purification of GAD has been successfully performed from four sources of the protein. Williams and Hager (1966) described the purification of GAD from Escherichia coli, where the enzyme is inducible when the organism is grown in the presence of glutamate (Strausbauch et. al., 1967). It was found that GAD could be isolated as a by-product during the actual purification of pyruvate dehydrogenase. The ammonium sulfate-precipitated GAD protein could then be dissolved in buffer and recrystallized to purity.

Another purification scheme was developed for GAD from the lobster central nervous system. Molinoff and Kravitz (1968) utilized a four-stage protocol to obtain a 40-fold purification of GAD. A post-mitochondrial extract was selectively precipitated between 40-65% $(\text{NH}_4)_2\text{SO}_4$, followed by gel filtration and ion exchange chromatography. The major proportion (about 90%) of the enzyme activity was found in the soluble 30,000 x g supernatant fraction.

A seven-step procedure for preparing highly purified GAD from the anaerobic bacterium, Clostridium perfringens, was developed by Cozzani (1970). A combination of MnCl_2 and $(\text{NH}_4)_2\text{SO}_4$ precipitations followed by gel filtration on Sephadex G-100 and G-200 resulted in a 24-fold purification. The homogeneity of the pure enzyme was established by starch-gel electrophoresis and sucrose-density gradient centrifugation.

The purification of GAD from mouse brain (Wu et. al., 1973) was accomplished after the discovery that a combination of AET and PyP could stabilize the enzyme for periods long enough to allow for a combination of $(\text{NH}_4)_2\text{SO}_4$ fractionation, gel filtration, calcium phosphate gel and DEAE-Sephadex chromatography (see Introduction). However, upon attempting this protocol for the purification of bovine GAD it was found that $(\text{NH}_4)_2\text{SO}_4$ precipitation steps resulted in a large loss of total GAD enzyme activity. This finding is consistent with the data (Fig. 14 and Table 2) presented in this thesis that the SO_4^{2-} ion is an extremely potent inhibitor of GAD. Because of this problem alternative methods of purification and stabilization were examined.

Two major differences exist between the Wu (1973) protocol and the method finally selected for use. The first was the use of DTT in place of AET as a sulfhydryl protective agent (see text). As mentioned earlier, although data would indicate otherwise (Roberts et. al., 1972), DTT (1 mM) was found to be a better stabilizer of enzyme activity than any other aminothiols or sulfhydryl compounds tested. The other

difference was the choice to use a 35,000 x g post-mitochondrial supernatant fraction. This decision was based on the finding that measurement of $^{14}\text{CO}_2$ evolution was not a valid estimate of true GAD activity if a mitochondrial fraction was being studied. Quantitation of $^{14}\text{CO}_2$ and $(^{14}\text{C})\text{GABA}$ produced in a isotopic assay from $(\text{U-}^{14}\text{C})\text{glutamic acid}$ indicates an increased $^{14}\text{CO}_2$ production upon addition of NAD^+ and CoA to assay mixtures, via the coupled reactions of glutamic acid dehydrogenase and α -ketoglutarate dehydrogenase (Drummond and Phillips, 1974). The addition of 1 mM AOAA to assays inhibited $(^{14}\text{C})\text{GABA}$ production 92% while $^{14}\text{CO}_2$ production was inhibited only 53% (Drummond and Phillips, 1974). Use of a mitochondria-containing fraction thus gives too high an estimate of GAD activity. This was the basis for the use of a post-mitochondrial fraction for a starting point for the present partial purification of bovine brain GAD. The study by Drummond and Phillips (1974) indicates that possibly 40% of all $^{14}\text{CO}_2$ being evolved and absorbed during the incubation could come from reactions occurring in the tricarboxylic acid cycle, specifically the decarboxylation of α -KG by α -ketoglutarate dehydrogenase. The use of a combination of AET and PyP by Wu *et. al.* (1973) as a stabilizer of GAD still resulted in a 99% loss of GAD specific enzyme activity; the present study, as previously mentioned, used DTT as a sulfhydryl compound and still experienced a 93% loss of GAD activity.

Attempts to utilize ammonium sulfate for concentrating protein (Wu *et. al.*, 1973) resulted in extremely large losses

in total GAD enzyme activity. As mentioned above the SO_4^{2-} anion is a potent inhibitor of both bovine and mouse brain GAD enzyme activity. Such losses in total enzyme activity prompted the use of a PM-30 Diaflow ultrafiltration membrane (see text) as the method of choice. This technique resulted in rapid concentration of column chromatography fractions with a minimal loss in GAD activity.

The examination of the most highly purified fraction of mouse brain GAD (Wu et. al., 1973) was performed using polyacrylamide gel disc electrophoresis. Prior to electrophoresis of GAD, the 3.5% acrylamide gel (containing Tris-HCl) was pre-electrophoresed with a Tris-glycine buffer. The purpose of this technique (to reduce the oxidizing agent, persulfate) is understandable, however it results in the actual electrophoresis of the GAD protein being performed in a continuous buffer system; this diminishes the resolution power of the method.

We felt that the use of a Tris-phosphoric acid buffer, replacing Tris-HCl, would eliminate the inhibitory Cl^- anion and that this same buffer (Tris-phosphoric acid) would be used for pre-electrophoresis of the separating gel. Thus, a discontinuous buffer system (Tris-glycine) could be employed for the final electrophoresis of GAD, affording increased resolution power. The problems concerning the analysis of polyacrylamide disc gel electrophoresis results, with reference to homogeneity of a protein band, has been discussed in the Results section.

Previous reports have indicated that acrylamide does not interfere with antibody production (Sussman et. al., 1968). This observation and those of Hartman and Udenfriend (1969) prompted a similar methodology for the induction of antibodies to bovine brain GAD. The electrophoresis of the partially purified bovine brain GAD preparation, followed by the slicing and re-electrophoresis of the GAD protein band allowed for the elimination of all contaminating proteins. Thus an electrophoretically pure protein was used for inoculation of rabbits.

Wu et. al. (1973) used their most highly purified soluble fraction of mouse brain GAD for inoculation into rabbits for the induction of anti-GAD serum (Saito and Roberts, 1974). When this anti-GAD serum was used for Ouchterlony double diffusion analysis a sharp precipitin band was observed with purified mouse GAD or a crude mouse brain preparation versus the antiserum. In the same study a comparison of Ouchterlony double diffusion tests with crude enzyme from various species was made. Single precipitin bands were observed with the anti-mouse GAD serum and crude brain preparations from rat, rabbit, guinea pig, quail, pigeon, and frog. However, the precipitin bands from the pigeon, quail, and frog preparations showed spurs. Thus, the enzymes from mammalian species were indistinguishable by Ouchterlony double diffusion analysis, however differences between the mouse enzyme and avian enzyme were detectable. When the inhibition of GAD enzyme activity (by antibody against mouse GAD) from these different species

was compared, it was found that the rat enzyme was inhibited somewhat less than the mouse enzyme. GAD from other species was inhibited to a much lesser extent. Microcomplement fixation studies of GAD from the various species indicated that the GAD from mouse, rat, and human were similar (Saito and Roberts, 1974). Saito and Roberts (1974) concluded that the mouse and rat enzyme resemble each other by double diffusion analysis, inhibition studies, and microcomplement fixation curves. The GAD of other mammalian species studied appeared to be indistinguishable from the mouse enzyme by double diffusion analysis, however the other techniques employed indicate that differences do exist between the enzymes.

Susz et. al. (1966), after a 158-fold partial purification of the mouse brain decarboxylase, noted that the enzyme is stable for one hour at 30° and 40° C, whereas a rapid loss of activity was noted at 50° C. The present study examines the thermal stability of a bovine brain, 4 day old mouse brain, and 20 day old mouse brain GAD. A rapid loss of GAD activity at 50° C was noted for both bovine and mouse (both ages) GAD. However, the loss of GAD activity was more extensive for the mouse enzyme than that reported by Susz et. al. (1966). Our data indicates (Fig. 11) that the bovine brain decarboxylase appears to be more stable at all temperatures studied than the mouse brain enzyme.

The optimal pH for GAD enzymic activity has been previously studied for several mouse brain preparations. Susz

et. al. (1966) reported the pH optimum of a crude GAD preparation (from mouse brain acetone powder) to be approximately 6.4, but that GAD activity remained essentially constant between 6.4-7.2; the most highly purified GAD preparation was maximally active at pH 7.2. The pH optimum of the highly purified GAD obtained by Wu and Roberts (1973) from adult Swiss albino mice gave a relatively sharp pH optimum around 7.0. For the Balb/c mouse strain, Wilson et. al. (1972) found that a pH of 6.8 yielded maximal GAD activity. These result compare favorably with a pH of 6.8, which in this study was found to yield maximal GAD activity for all extracts.

The pH optimum conditions for GAD from other sources has also been determined for: the anaerobic bacterium Clostridium perfringens, 4.7 (Cozzani et. al., 1970); GAD from the lobster CNS, 8.0 (Molinoff and Kravitz, 1968); the slime mold Physarum polycephalum, 5.6-6.0 (Nations and Anthony, 1969).

We cannot survey here the enormous literature relating to the effect of various anions and/or cations on the GAD/GABA system. The results of this study concerning the inhibition of GAD enzyme activity by anions (Fig. 13 and 14, Table 2) are in agreement with Susz et. al. (1966) who indicate that GAD (mouse) inhibition by anions is in the order $I^- > NO_3^{2-} > SO_4^{2-} > Br^- > Cl^- > C_2H_3O_2^- > F^-$. The same study (Susz et. al., 1966) also showed the inhibition of GAD activity by Cl^- ions to be competitive with substrate. Interestingly, the bacterial enzyme (Shukuya and Schwert, 1960) differs from the brain enzyme by being activated by anions.

Chloride ions could have a physiological function of

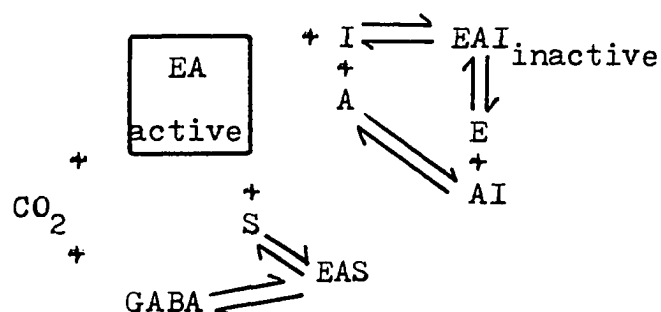
regulating brain GAD activity. The role of glutamate as a potential excitatory compound in both vertebrate and invertebrate synaptic clefts, and GABA (which is made from glutamate by GAD) as a putative inhibitory neurotransmitter has been discussed in the Introduction. Since the actual values for extracellular (0.1 M) and intracellular (0.02 M) concentrations of Cl^- ion also fall within a range of concentration that affect GAD activity in the present study, it is conceivable that variations in Cl^- ion concentration at the pre-synaptic nerve terminal could control the relative ratio of glutamate: GABA by the regulation of GAD enzyme activity. Thus, the predominant compound to be released into the synaptic cleft upon stimulation of the pre-synaptic nerve terminal (see Srinivasan et. al., 1969, concerning the efflux of ^3H -GABA from brain slices) could be regulated by Cl^- ion concentration. Thus the Cl^- ion concentration could determine whether a nerve ending, when stimulated, has an excitatory (glutamate release) or inhibitory (GABA release) function.

Two previous studies support the above theory for the regulation of the rate of transmitter biosynthesis by Cl^- ion. Potter et. al. (1968) has shown that the biosynthesis of acetylcholine, even though regulated by feedback control of acetylcholine acetylase, may also be influenced by the ionic composition of the axoplasm. Also, a neurophysiological study (Kandel et. al., 1967) on Aplysia californica (a marine mollusc) ganglion showed that the terminal branches of a single cholinergic neuron produce both inhibitory and excitatory effects

on different post-synaptic cell membranes.

Extensive experiments have been conducted on the inhibition of GAD by substances which react with sulfhydryl groups, and by inhibitors that combine with the aldehyde moiety of the coenzyme, pyridoxal-5'-phosphate (see articles: Roberts and Simonsen, 1963; Tapia *et. al.*, 1966; Tapia and Awapara, 1969; Tapia and Sandoval, 1971). Shukuya and Schwert (1960) in a study of the bacterial decarboxylase, suggest that it is a sulfhydryl enzyme (also see Strausbauch and Fischer, 1970, for a sulfhydryl group titration study). The sensitivity to oxygen and protection by GSH, DTT, or 2-mercaptoethanol of the brain decarboxylase (Roberts *et. al.*, 1972) suggest it too contains a sensitive sulfhydryl group, the physical integrity of which is essential for the maintenance of enzymatic activity.

The complex kinetics of reactions catalyzed by PyP requiring enzymes has been considered by Roberts and Simonsen (1963) in the following reaction sequence: A--PyP; E--GAD; I--inhibitor; S--glutamate.



The inhibitor compounds considered in this study react with the sulfhydryl groups in GAD, each in a specific manner: iodoacetamide is an alkylating agent; p-hydroxymercuribenzoic acid is a mercaptide forming reagent; 1,2-napthoquinone-4-sulfonic acid and nitroso R salt form adducts with sulfhydryl groups (see Roberts and Simonsen, 1963, for detailed discussion of inhibitors). Matsuo and Greenberg (1959) have shown that the final two compounds are also inhibitors of cystathionase, another PyP requiring enzyme. That these final two compounds inhibit GAD in a competitive manner (Matsuo and Greenberg, 1959) with respect to substrate concentration suggest that a sulfhydryl moiety may be at or near the enzyme's active site.

Another much larger group of inhibitors of PyP requiring enzymes are the carbonyl trapping agents which inhibit enzyme activity by combining with the aldehyde group on the coenzyme, pyridoxal-5'-phosphate. For a thorough discussion on the inhibition of the decarboxylase by AOAA, hydroxylamine, and related compounds see the articles cited at the beginning of this section.

The changes in GAD enzyme activity during postnatal development in mouse brain are paralleled by rapid alterations in brain morphology. At birth, the process of differentiation of neuroblast to neuron is largely completed. During the first postnatal week a transition occurs from a time when differentiation is the major process occurring to a period when growth and maturation of neuronal elements predominates. The second

and third weeks of postnatal brain development are marked by increases in the size of cell bodies which are now becoming rapidly separated by expanding dendritic processes. There also appears an increased prominence of Nissl granules, a corresponding decrease in nuclear DNA, and increased myelination of the axons of major fiber tracts. These changes occur throughout the whole brain but are most noticeable moving from hindbrain toward the cerebral hemispheres. For a review of the development of the nervous system see *The Neurosciences*, second study program (Schmitt, 1970).

Comparisons of postnatal morphological changes in rat brain with those of mouse reveal that the two species are similar, allowing comparisons of enzymatic activity changes between the species. Sugita (1917) compiled a detailed and precise timetable for morphological changes in rat brain; during the first 10 postnatal days significant increases occur in cortical thickness, lamination and cell number. In the rat, the time period of greatest increase in GAD levels is during the second and third week of postnatal brain development (Van Den Berg *et. al.*, 1965; Bayer and McMurray, 1967; Sims and Pitts, 1970). During the first month of postnatal life GAD activity in rat brain increased approximately 10-fold (Sims and Pitts, 1970). The increase in GAD specific enzyme activity presented in this study is nearly identical to that reported by Sims and Pitts (1970).

The initial rapid increase in GAD enzyme levels, which begins by day 6 (see Figs. 16 and 18) precedes a burst of

whole brain protein synthesis which begins on day 11 (Pitts and Quick, 1967). The rate of brain RNA synthesis begins to increase by day 6 (Balazs et. al., 1968) and thus the accelerated synthesis of GAD and SSA-DH (Pitts and Quick, 1967), which begin to rise at day 6, are among the first enzymes to reflect this increased amount of RNA.

Potter et. al. (1945) measured SSA-DH and ATPase activity in developing rat brain; the general form of the GAD activity profile in our study closely resembles the enzyme activity curves in their study. The activity of acetylcholinesterase (Cohn and Richter, 1956) and ATPase (Samson and Quinn, 1967) have both been shown to increase rapidly prior to the 10th postnatal day in rat brain. SSA-DH (Pitts and Quick, 1967) and GABA-T (Sims et. al., 1968) enzyme activity profiles each follow a characteristic course during the development of the rat brain.

It appears, therefore, that the period of maximum increase in glutamic acid decarboxylase activity is correlated to the period of maturation of the nuclear masses and fiber tracts of the central nervous system.

The correlation of changes in GAD activity with GABA levels has been examined in a single brain structure, the chick optic lobe, during embryonic development and post-hatching (Sisken et. al., 1961). At the 7-day embryo stage the levels of GAD and GABA are among the first detectable and increases in GAD activity are proportional to increases in GABA levels; mature levels of enzyme and product are attained

between 5-10 days post-hatching. Roberts and Kuriyama (1968) have shown in chick cerebellum that GAD activity increased most rapidly just prior to hatching, a period during which the cerebellum experiences the most rapid proliferation of dendritic growth.

A unique feature of the work presented here is that it is the first study to examine whether the increase in GAD enzyme activity observed over mouse brain development (Fig. 16) represents an increase in the absolute amount of GAD enzyme protein. The quantitative immunoprecipitation of GAD in developing mouse brain by rabbit anti-bovine GAD antisera indicates that the amount of protein in an immune precipitate per brain increases 10 fold over the period between 1-28 days postnatally (Fig. 18). Such an increase closely coincides with the GAD enzyme activity profile (Fig. 16). Therefore, we conclude that the increase in GAD specific enzyme activity over the development of mouse brain represents an increase in the absolute amount of GAD enzyme protein.

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